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Office Action Summary

Application No.

10/003,846

Applicant(s)

KNUDSEN ET AL

Examiner

Claire M. Kaufman

Art Unit

1646

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If the period for reply specified above is less than thirty (30) days, a reply within the statutory minimum of thirty (30) days will be considered timely.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

- 1) ☒ Responsive to communication(s) filed on 04 December 2001.
- 2a) ☐ This action is **FINAL**. 2b) ☒ This action is non-final.
- 3) ☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

- 4) ☒ Claim(s) 1-43, 46 and 47 is/are pending in the application.
- 4a) Of the above claim(s) 46 and 47 is/are withdrawn from consideration.
- 5) ☐ Claim(s) _____ is/are allowed.
- 6) ☒ Claim(s) 1-43 is/are rejected.
- 7) ☐ Claim(s) _____ is/are objected to.
- 8) ☒ Claim(s) 1-43, 46 and 47 are subject to restriction and/or election requirement.

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Application Papers

- 9) ☒ The specification is objected to by the Examiner.
- 10) ☐ The drawing(s) filed on _____ is/are: a) ☐ accepted or b) ☐ objected to by the Examiner.
Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).
Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).
- 11) ☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

- 12) ☒ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).
- a) ☒ All b) ☐ Some * c) ☐ None of:
1. ☒ Certified copies of the priority documents have been received.
 2. ☐ Certified copies of the priority documents have been received in Application No. _____.
 3. ☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

- 1) ☒ Notice of References Cited (PTO-892)
- 2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
- 3) ☒ Information Disclosure Statement(s) (PTO-1449 or PTO/SB/08)
Paper No(s)/Mail Date 03/07/02, 10/09/02
- 4) ☐ Interview Summary (PTO-413)
Paper No(s)/Mail Date. _____
- 5) ☐ Notice of Informal Patent Application (PTO-152)
- 6) ☐ Other: _____

DETAILED ACTION

The preliminary amendment filed 12/04/01 has been entered.

Election/Restrictions

Restriction to one of the following inventions is required under 35 U.S.C. 121:

- I. Claims 1-43, drawn to method of identifying a compound interacting with a G protein-coupled receptor, classified in class 435, subclass 7.2.
- II. Claims 46-47, drawn to method of producing a pharmaceutical preparation, classification dependent on compound structure, for example, classified in class 514, subclass 2.

The inventions are distinct, each from the other because of the following reasons:

Inventions I and II are related as in that both use the method of identifying a GPCR interacting compound, but because II requires a compound instead of simply an assay, the inventions are distinct. The invention of Group I does not require the particulars of the Group II as claimed for patentability, and the method of making the pharmaceutical preparation could have utility by itself as an effector of a GPCR.

Because these inventions are distinct for the reasons given above, have acquired a separate status in the art as shown by their different classification and each invention requires a separate non-coextensive search, restriction for examination purposes as indicated is proper.

During a telephone conversation with Dr. Reza Green on February 17, 2004, a provisional election was made with traverse to prosecute the invention of Group I, claims 1-43. Affirmation of this election must be made by applicant in replying to this Office action. Claims 46-47 are withdrawn from further consideration by the examiner, 37 CFR 1.142(b), as being drawn to a non-elected invention.

Applicant is reminded that upon the cancellation of claims to a non-elected invention, the inventorship must be amended in compliance with 37 CFR 1.48(b) if one or more of the currently named inventors is no longer an inventor of at least one claim remaining in the application. Any amendment of inventorship must be accompanied by a request under 37 CFR 1.48(b) and by the fee required under 37 CFR 1.17(i).

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Specification

The disclosure is objected to because of the following informalities: on page 22, line 7, "GPCR/BHK cells?/".

Appropriate correction is required.

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Claim Objections

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Claim 4 is objected to because of the following informalities: a hyphen is present in "phosphorylation-independent" in line 4, but does not occur elsewhere in claims (*e.g.*, claim 13 or 25). Appropriate correction is required.

Claim Rejections - 35 USC § 102

The following is a quotation of the appropriate paragraphs of 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

A person shall be entitled to a patent unless –

(b) the invention was patented or described in a printed publication in this or a foreign country or in public use or on sale in this country, more than one year prior to the date of application for patent in the United States.

Claims 1-3, 9, 10-12, 18, 22-24, 31-33 and 39 are rejected under 35 U.S.C. 102(b) as being anticipated by US Patent 5,891,646 (cited by Applicants).

US Patent 5,891,646 (Barak et al., '646) teaches a method of identifying compounds capable of initiating signaling of or deactivating a GPCR (β 2adrenergic receptor) by contacting HEK cell membranes comprising the GPCR with β arrestin2-GFP, visually separating GPCR-bound and -unbound arrestin, and determining the level of GPCR-bound arrestin. The test compound, isoproterenol, acted as an agonist, mediating an increase in the bound compared to unbound arrestin as was seen by enhanced membrane arrestin fluorescence with concomitant loss of cytosolic fluorescence, such that the arrestin distribution shifted to the membrane. Agonist exposure caused an increase of ten-fold compared to control (distribution prior to agonist exposure) (col. 19, lines 48-63). The same experiment was conducted with the GPCR antagonist propanol (*e.g.*, col. 20, lines 19-30) with the opposite results, *i.e.*, more unbound than bound was seen. '646 is silent with respect to kinase presence; however, because wildtype arrestin will not bind unphosphorylated GPCR (*e.g.*, col. 1, lines 40-42) and phosphorylation requires a kinase,

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the kinase was necessarily present in the cell. Also taught is the method with addition of GRK kinase (col. 9, lines 38-42 and Fig. 5). The same method was conducted with an added carrier, mouse monoclonal antibody directed against an 12CA5(HA) tag of the β_2 adrenergic receptor, which bound the cell membrane indirectly through binding of the membrane-associated translocated GPCR (col. 20, lines 50-62).

Note that because the claims are drawn to a method "comprising" and because the cell membrane of part (a) of the instant claims is not necessarily isolated, the instant invention reads on using whole cells.

Claims 1-19, 22-40 and 43 are rejected under 35 U.S.C. 102(b) as being anticipated by Kovoov et al. (J. Biol. Chem., 274(11):6831, 1999, cited by Applicants).

Kovoov et al. teach a method of identifying a compound capable of initiating signaling of or deactivating a GPCR by contacting membranes (liposomes) containing β_2 adrenergic receptor (β AR) phosphorylated by a GRK (also known as β ARK) with wildtype arrestin or R169E or 1-382 arrestin mutants. The arrestin was labeled with tritium, and that which bound a Sepharose column and to receptor-containing membranes was separated and quantified by liquid scintillation (legend of Fig. 1). The compound tested was isoproterenol.

Note that the method is the same for identifying an agonist or antagonist, and determination of whether the test compound is an agonist or antagonist by looking at levels is a mental step.

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Claim Rejections - 35 USC § 103

The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

(a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negated by the manner in which the invention was made.

This application currently names joint inventors. In considering patentability of the claims under 35 U.S.C. 103(a), the examiner presumes that the subject matter of the various claims was commonly owned at the time any inventions covered therein were made absent any

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evidence to the contrary. Applicant is advised of the obligation under 37 CFR 1.56 to point out the inventor and invention dates of each claim that was not commonly owned at the time a later invention was made in order for the examiner to consider the applicability of 35 U.S.C. 103(c) and potential 35 U.S.C. 102(e), (f) or (g) prior art under 35 U.S.C. 103(a).

Claims 2, 4-8, 11, 13-17, 23, 25-29, 32 and 34-38 are rejected under 35 U.S.C. 103(a) as being unpatentable over US Patent 5,891,646 and further in view of Kovoov et al. (J. Biol. Chem., 274(11):6831, 1999) and Palczewski (Eur. J. Biochem., 248:261-269, 1997).

US Patent 5,891,646 is relied upon for the teaches above. US Patent 5,891,646 does not teach a phosphorylation-independent arrestin mutant or, specifically, GRK.

Kovoov et al. teach two phosphorylation independent β arrestin mutants: one which is R169E and another with position 383 being a stop codon so that it comprises 1-382. These arrestins are constitutively active and *in vitro* bind the agonist activated β_2 adrenergic receptor regardless of its phosphorylation status. They also bind δ opiod receptor. Also taught is the model for signaling by diverse GPCRs (p. 6831, beginning last sentence of col. 1). "According to the model, activated receptor is first phosphorylated by a G protein-coupled receptor kinase (GRK). An arrestin protein binds to the activated phosphoreceptor, thereby blocking G protein interaction..." forming an "arrestin-receptor complex". The discussion of effector molecules (col. 2, beginning middle of first paragraph) says there are only six mammalian GRKs and four arrestins that have been found so far. "This suggests that at least some of the kinases and arrestins regulate numerous receptors. Thus, these proteins are attractive targets for research designed to delineate common molecular mechanisms underlying the regulation of GPCR signaling in cells (and to create fairly universal tools for the experimental and/or therapeutic intervention in the process)."

It would have been obvious at the time the invention was made to practice the assay of '646 with a phosphorylation-independent arrestin mutant taught by Kovoov et al. to avoid the need of a kinase and because Kovoov teaches that "...these proteins [*i.e.*, arrestins] are attractive targets for research designed to delineate common molecular mechanisms underlying the regulation of GPCR signaling in cells..." Further, even if '646 did not use a GRK, one would have been motivated to use a GRK because Kovoov et al. teach that GRKs are responsible for phosphorylating GPCRs so that arrestins can bind.

Claims 19-21 and 40-43 are rejected under 35 U.S.C. 103(a) as being unpatentable over Kovoov et al. (J. Biol. Chem., 274(11):6831, 1999) and US Patent 5,670,360 (Thorens).

Kovoov et al. teach a method of identifying a compound capable of initiating signaling of or deactivating a GPCR by contacting membranes (liposomes) containing β_2 adrenergic receptor (β_2 AR) phosphorylated by a GRK (also known as β ARK) with wildtype arrestin or R169E or 1-382 arrestin mutants. The arrestin was labeled with tritium, and the arrestin which bound to a Sepharose column and to receptor-containing membranes was separated and quantified by liquid scintillation (legend of Fig. 1). The compound tested was isoproterenol. Also taught is recombinant expression of β_2 AR and opiod receptor GPCRs in *Xenopus* oocytes (p. 6832, col. 1, beginning of first paragraph). Kovoov does not teach using SPA (scintillation proximity assay) beads or WGA (wheatgerm agglutinin).

US Patent 5,670,360 ('360) teaches an assay to identify a compound capable of activating or deactivating the GPRC GLP-1 receptor by means of a high throughput screening assay using SPA (scintillation proximity assay) beads coated with WGA (wheatgerm agglutinin). The WGA allowed GPL1- receptor bearing membranes to be immobilized on SPA beads. The membranes were prepared by recombinant cloning of the receptor into the CHL cell line (col. 11, lines 5 through col. 12, line 28).

It would have been obvious at the time the invention was made to practice the method of Kovoov by substituting SPA beads coated with WGA for the sepharose column to conduct the liquid scintillation assay since the SPA/WGA beads were commercially available and convenient to use in an old and routine assay method (e.g., col. 5, lines 40-47). It further would have been obvious to transform a cell line with the a GPCR cDNA of Kovoov et al. as a source of GPCR-containing membranes in the assay instead of liposome membranes in order to better understand the association of GPCR with endogenous membrane-associated molecules and to a virtually endless supply of membranes from routinely cultured cells.

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Prior Art

The prior art made of record and not relied upon is considered pertinent to applicant's disclosure. Krupnic et al. (Annu. Rev. Pharmacol. Toxicol., 38 :289,1998) describes the relationship of GPCRs, arrestins and kinases. Also listed are common G protein-coupled receptor kinase names, for example β ARK (Table 1 on p. 293). Palczewski (Eur. J. Biochem., 248:261-269, 1997) describes the relationship of GPCRs to GRKs. Mundell et al.(Biochem., 38:8723-8732, 10 June 1999) describe transfection of HEK293-EBNA cells with one of several GPCRs: β_2 AR, m2 and m3 muscarinic acetylcholine receptors (p. 8725, last two full paragraphs of col. 1).

Conclusion

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Claire M. Kaufman, whose telephone number is (571)272-0873. Dr. Kaufman can generally be reached Monday, Tuesday and Thursday from 8:30AM to 2:30PM.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Yvonne Eyler, can be reached at (571)272-0871.

Any inquiry of a general nature or relating to the status of this application should be directed to the Group 1600 receptionist whose telephone number is (703) 308-0196.

Claire M. Kaufman, Ph.D.



Patent Examiner, Art Unit 1646

February 17, 2004

Notice of References Cited	Application/Control No. 10/003,846	Applicant(s)/Patent Under Reexamination KNUDSEN ET AL.	
	Examiner Claire M. Kaufman	Art Unit 1646	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
	A	US-5,670,360	09-1997	Thorens, Bernard	
	B	US-			
	C	US-			
	D	US-			
	E	US-			
	F	US-			
	G	US-			
	H	US-			
	I	US-			
	J	US-			
	K	US-			
	L	US-			
	M	US-			

FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	Classification
	N					
	O					
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	Krupnic et al., The role of receptor kinases and arrestins in G protein-couple receptor regulation, Annu. Rev. Pharmacol. Toxicol., 38:289-319, 1998.
	V	Palczewski et al., GTP-binding-protein-coupled receptor kinases: Two mechanistic models, Eur. J. Biochem., 248:261-269, 1997.
	W	Mundell et al., Characterization of G protein-coupled receptor regulation in antisense mRNA-expressing cells with reduced arrestin levels, Biochem., 38:8723-8732, 10 June 1999.
	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

Characterization of G Protein-Coupled Receptor Regulation in Antisense mRNA-Expressing Cells with Reduced Arrestin Levels[†]

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Received February 15, 1999; Revised Manuscript Received May 20, 1999

ABSTRACT: Previous studies with overexpressing wild-type or dominant negative nonvisual arrestins have established a role for these proteins in β_2 -adrenergic receptor (β_2 AR) internalization, desensitization, and resensitization. To validate and extend such findings, we employed an antisense strategy to target the nonvisual arrestins, arrestin-2 and arrestin-3, and determined the associated effects on the regulation of G protein-coupled receptor (GPCR) signaling. HEK293 cells stably expressing antisense constructs targeting arrestin-2 exhibited a selective reduction ($\sim 50\%$) in arrestin-2 levels, while arrestin-3 antisense constructs resulted in reductions ($\geq 50\%$) in both arrestin-2 and arrestin-3 levels. Initial analysis of these cells demonstrated that a reduced level of arrestin expression resulted in a significant decrease in the extent of agonist-induced internalization of exogenously expressed β_2 ARs, but had no effect on internalization of either m2 or m3 muscarinic acetylcholine receptors. Additional characterization involved assessing the role of arrestins in the regulation of endogenous GPCRs in these cells. Reduced arrestin levels significantly decreased the rate of endogenous β_2 AR internalization, desensitization, and resensitization. Further analysis demonstrated that the desensitization of endogenous A_{2b} adenosine and prostaglandin E_2 -stimulated receptors was also attenuated in cells with reduced arrestin levels. The effects on the β_2 -adrenergic, A_{2b} adenosine, and PGE_2 -stimulated receptors were similar among cell lines that exhibited either a selective reduction in arrestin-2 levels or a reduction in both arrestin-2 and -3 levels. These findings establish the utility of antisense approaches in the examination of arrestin-mediated GPCR regulation.

Prolonged exposure of G protein-coupled receptors (GPCRs)¹ to agonist often results in a rapid decrease of receptor responsiveness, a process termed desensitization (1). Mechanisms mediating GPCR desensitization include agonist-dependent phosphorylation by G protein-coupled receptor kinases (GRKs), which in turn promotes high-affinity binding of arrestins (1). Arrestin binding sterically inhibits G protein interaction with the agonist-activated GPCR (2, 3).

There are currently four cloned arrestin family members. Arrestin-1 or visual arrestin is expressed predominantly in rod cells and acts by preventing light-activated phosphorylated rhodopsin from interacting with transducin (4). Arrestin-4 (X-arrestin or arrestin-C) is specifically expressed in cone cells, suggesting a role in regulating cone phototransduction (5, 6). The ubiquitously expressed arrestin-2 (β -arrestin-1) (7) and arrestin-3 (β -arrestin-2) (8, 9) likely

regulate the interaction of a wide variety of GPCRs with their corresponding G proteins. Indeed, overexpression studies have demonstrated a role for nonvisual arrestins in the desensitization of several GPCRs, including the β_2 -adrenergic (10), β_1 -adrenergic (11), and α_{1B} -adrenergic (12) receptors.

An additional facet of GPCR regulation involves internalization of the receptor following agonist exposure (13). Internalization may play divergent roles for different GPCRs. For example, while internalization plays a key role in the resensitization of the β_2 -adrenergic receptor (β_2 AR) (14–16), it appears to prolong desensitization of the m4 muscarinic acetylcholine receptor (m4AChR) (17). Inhibition of internalization has also been shown to differentially affect the desensitization and resensitization of A_{2a} adenosine and secretin receptor responsiveness while having no effect on IP-prostanoid responses (18). Recent studies have shown that arrestin-2 and arrestin-3 can facilitate internalization of the β_2 AR (19, 20) and other GPCRs (13). Indeed, the extent of agonist-mediated internalization of the β_2 AR in several different cell lines correlates with the endogenous GRK and arrestin levels (21). Mechanistic insight into this process has revealed that nonvisual arrestins can bind to both receptors and clathrin and thus can function as adaptor proteins to mediate GPCR uptake into clathrin-coated pits (20, 22).

Studies to date have employed overexpression of either wild-type (19, 20) or dominant negative (19, 23, 24) arrestins to elucidate many of the functions of these proteins. For

[†] This work was supported in part by National Institutes of Health Grant GM47417 and an Established Investigatorship from the American Heart Association.

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¹ Abbreviations: mAChR, muscarinic acetylcholine receptor; β_2 AR, β_2 -adrenergic receptor; BSA, bovine serum albumin; GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; HEK293 cells, human embryonic kidney cells; Iso, (–)-isoproterenol; PBS, phosphate-buffered saline.

example, arrestin-2 and arrestin-3 dominant negative mutants that bind well to clathrin but lack GPCR binding activity effectively inhibit agonist-induced internalization of the β_2 -AR in HEK293 cells (23, 24). While these studies have provided important insight into arrestin function, one of the inherent drawbacks with this approach is the potential for nonspecific effects associated with protein overexpression in a cell. An alternative and perhaps preferable approach is the use of antisense oligodeoxynucleotide strategies to target specific mRNAs and reduce protein expression. Antisense approaches have been successfully used to selectively decrease a number of cellular signaling proteins, including GPCRs (25), G proteins (26), and GRKs (27). However, an effective arrestin antisense construct has not been reported.

In the study presented here, we systematically characterized the effectiveness of various arrestin-2 and arrestin-3 antisense constructs. Two candidate antisense constructs that reduced the extent of arrestin overexpression in a transient expression system were then stably transfected into HEK293 cells, where their ability to reduce endogenous arrestin levels and effect GPCR regulation was investigated.

EXPERIMENTAL PROCEDURES

Materials. Human embryonic kidney cells transformed with the EBNA vector (HEK293-EBNA) were purchased from Invitrogen. Tissue culture media and fetal bovine serum were obtained from Life Technologies, Inc. Fugene-6 transfection agent, hygromycin, and Geneticin were from Boehringer Mannheim. Anti-mouse immunoglobulin conjugated with alkaline phosphatase and goat anti-mouse and anti-rabbit immunoglobulin conjugated with horseradish peroxidase were purchased from Bio-Rad. [125 I]Pindolol, [125 I]-labeled cyclic AMP, [3 H]-N-methylscopolamine (NMS), and [3 H]-labeled cyclic AMP were purchased from NEN Life Science products. All other reagents were from Sigma.

Generation of Antisense Expression Constructs. Full-length and truncated arrestin antisense constructs for transient transfection were generated by PCR amplification followed by subcloning into pcDNA3. A full-length human arrestin-3 antisense construct was amplified using sense (5' CAA TGG ATC CAT GGG GGA GAA ACC CGG GAC CAG G 3') and antisense (5' CAA TGG ATC CTC AGC AGA GTT GAT CAT CAT AGT CGT CAT C 3') primers that contained *Bam*HI restriction sites at the 5' end. The resulting product was digested with *Bam*HI, subcloned into *Bam*HI-digested and phosphatase-treated pcDNA3, and then assessed for orientation. Truncated arrestin-3 antisense constructs were generated using the arrestin-3 sense primer and antisense primers that contained a *Hind*III site at the 5' end: base pairs 1–801 (5' CAA TAA GCT TGG AGC TGG GAG ATA CCT GGT C 3'), 1–402 (5' CAA TAA GCT TTG GGC CTG GCT GCA GTG TGA C 3'), and 1–102 (5' CAA TAA GCT TTT TGT CCA GGT GAT CTA CGA A 3'). To generate arrestin-2 antisense constructs, a human arrestin-2 cDNA was first digested with *Hind*III and subcloned into pcDNA3. Untranslated sequences were removed by PCR using sense (5' CAA TTC TAG AAT GGG CGA CAA AGG GAC G 3') and antisense (5' CAA TCT CGA GCT ATC TGT CGT TGA GCC GCG G 3') primers that contained *Xba*I and *Xho*I sites adjacent to the start and stop codons,

respectively. Truncated arrestin-2 constructs were generated using the arrestin-2 sense primer and one of the following antisense primers: base pairs 1–801 (5' CAA TCT CGA GCG TCG AGC TGG GTG CCA CAG T 3', *Xho*I site at the 5' end), 1–483 (5' CCA CAG AAT TCC GCT TGT GGA TCT TCT CCT C 3', *Eco*RI site at the 5' end), and 1–134 (5' CAA TGG ATC CAC CAG GAC CAC ACC ATC CAC A 3', *Bam*HI site at the 5' end).

Antisense constructs for stable expression were created in the mammalian expression vector pREP4. The pREP4–arrestin-3 antisense construct was made by excising the ~0.8 kb *Hind*III–*Bam*HI insert from pcDNA3–arrestin-3 (base pairs 1–801) and subcloning into *Hind*III–*Bam*HI-digested pREP4. Similarly, the 0.8 kb insert from pcDNA3–arrestin-2 (base pairs 1–801) was excised with *Xho*I and *Xba*I, blunted with Klenow, and then subcloned into *Pvu*II-digested pREP4.

Cell Culture and Transfection. HEK293 and COS-1 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 units/mL penicillin G, and 100 μ g/mL streptomycin sulfate (complete medium) at 37 °C in a humidified atmosphere of 95% air and 5% CO₂. HEK293-EBNA cells were always maintained in the presence of Geneticin (200 μ g/mL) to maintain EBNA vector expression.

For transient transfections, cells were grown in 100 mm dishes to 80–90% confluency and transfected with 1–10 μ g of DNA using Fugene-6 following the manufacturer's instructions. Briefly, HEK293 cells were incubated with a DNA/Fugene mixture for 24 h; the medium was then replaced, and the cells were analyzed 48 h after transfection. In experiments with COS-1 cells, the cells were transfected with 3 μ g of total DNA using Lipofectamine following the manufacturer's instructions.

For stable transfections, 4 μ g of DNA was used to transfect HEK293-EBNA cells. Three days after transfection, cells were diluted and replated in complete medium supplemented with 400 μ g/mL hygromycin and 200 μ g/mL Geneticin. The medium was subsequently replaced every 3 days, and surviving colonies were expanded into individual clonal lines.

Western Blot Analysis. Cells were lysed by addition of 200–500 μ L of ice-cold lysis buffer [20 mM HEPES (pH 7.4), 200 mM NaCl, 10 mM EDTA, 1% Triton X-100, 0.2 mg/mL benzamidine, 0.1 mg/mL leupeptin, and 0.5 mM phenylmethanesulfonyl fluoride] to cell monolayers. Insoluble material was pelleted by centrifugation in a microcentrifuge at 13 000 rpm for 3 min at 4 °C, and the resulting supernatant was aliquoted, snap-frozen in liquid nitrogen, and stored at –70 °C. Supernatants (40 μ g of total protein) were electrophoresed on a 10% SDS–polyacrylamide gel according to the method of Laemmli (28). Protein was then transferred to nitrocellulose and incubated with either arrestin-2 or arrestin-3 selective rabbit polyclonal antibodies made against GST fusion proteins containing either the C-terminal 62 amino acids of bovine arrestin-2 (residues 357–418) or 60 amino acids of bovine arrestin-3 (residues 350–409). Blots were then incubated with a goat anti-rabbit immunoglobulin conjugated with horseradish peroxidase and visualized by ECL detection according to the manufacturer's instructions.

β_2 AR Binding and Ligand Competition Assays. To determine the β_2 AR density in control and transfected cells, whole cells were harvested in ice-cold phosphate-buffered saline

(PBS). Two hundred micrograms of cell protein was then incubated in PBS containing 0.3 nM [125 I]iodopindolol with or without 1 μ M (–)-alprenolol (to estimate total receptor number) or 100 nM CGP-12177 (to estimate surface receptor number) for 3 h at 14 °C. All binding reactions were terminated by the addition of 5 \times 4 mL of ice-cold 25 mM Tris (pH 7.5) and 2 mM MgCl₂ followed by rapid filtration through Whatman GF/C filters using a Brandel cell harvester. Protein concentrations were determined using a Bio-Rad protein assay with bovine serum albumin (BSA) as a standard.

Internalization Assays in Cells Overexpressing Flag-Tagged β_2 AR. The extent of β_2 AR internalization was measured by ELISA as described by Daunt et al. (29). Briefly, cells plated at a density of 6×10^5 cells per 60 mm dish were transfected with 5 μ g of pcDNA3-Flag- β_2 AR, and then split after 24 h of transfection into 24-well tissue culture dishes coated with 0.1 mg/mL poly-L-lysine. Twenty-four hours later, cells were incubated with DMEM containing 300 μ M ascorbic acid [with or without 0.001–10 μ M (–)-isoproterenol (Iso)] for 0–60 min at 37 °C. Reactions were stopped by removing the medium and fixing the cells with 3.7% formaldehyde in TBS [20 mM Tris (pH 7.5), 150 mM NaCl, and 20 mM CaCl₂] for 5 min at room temperature. Cells were washed three times with TBS, incubated for 45 min with TBS containing 1% BSA, and then incubated with a primary antibody (anti-Flag monoclonal M1, 1:1000 dilution in TBS/BSA) for 1 h at room temperature. Cells were washed three times with TBS, reblocked with TBS/BSA for 15 min at room temperature, and then incubated with secondary antibody (goat anti-mouse conjugated with alkaline phosphatase, 1:1000 dilution in TBS/BSA) for 1 h at room temperature. Cells were washed three times with TBS, and a colorimetric alkaline phosphatase substrate was added. When adequate color change was achieved, 100 μ L of sample was added to 100 μ L of 0.4 M NaOH to terminate the reaction, and the samples were read at 405 nm using a microplate reader.

Internalization of Endogenous β_2 ARs. Cells on 100 mm dishes (80–90% confluency) were incubated with medium containing 300 μ M ascorbic acid with or without 0.01–10 μ M Iso at 37 °C for 0–60 min. Cells were then washed three times in ice-cold PBS and surface receptor levels determined in whole cells by ligand binding with [125 I]-iodopindolol with or without 100 nM CGP-12177 at 14 °C as described above.

Internalization of m2 and m3 Muscarinic Acetylcholine Receptors. Cells were transiently transfected as described above with 10 μ g of pcDNA3 containing either the m2- or m3AChR. One day post-transfection, cells from 100 mm plates were passaged onto 60 mm plates for use the following day. Cells were incubated in the presence or absence of 1 mM carbachol for 0–60 min and then washed with 3 \times 10 mL of ice-cold PBS at 4 °C. Two hundred micrograms of cell protein was then incubated with 2 nM [3 H]NMS with or without 10 μ M atropine for 3 h at 4 °C to assess cell surface mAChR density. Binding reactions were terminated by the addition of 5 \times 4 mL of ice-cold 25 mM Tris (pH 7.5) and 2 mM MgCl₂ followed by rapid filtration through Whatman GF/C filters using a Brandel cell harvester.

Adenylyl Cyclase Assays. Vehicle or agonist was added directly to the culture medium for varying times. Cells were

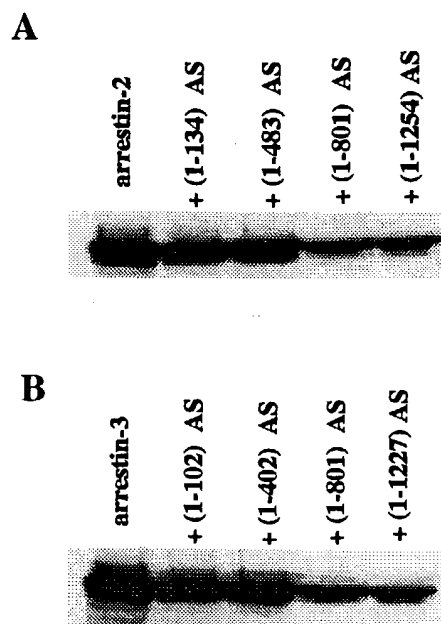


FIGURE 1: Coexpression of different length arrestin antisense mRNAs with wild-type arrestin-2 or arrestin-3 in COS-1 cells. Cells were transiently transfected with (A) 1 μ g of pcDNA3–arrestin-2 and either 2 μ g of GRK2 (495–689) (control) or 2 μ g of different length arrestin-2 antisense constructs in pcDNA3 or (B) 1 μ g of pcDNA3–arrestin-3 and either 2 μ g of GRK2 (495–689) (control) or 2 μ g of different length arrestin-3 antisense constructs in pcDNA3. Whole cell lysates were subjected to SDS–polyacrylamide gel electrophoresis followed by electrophoretic transfer and immunoblotting with rabbit polyclonal antibodies selective for arrestin-2 (A) or arrestin-3 (B). Detection of protein expression was performed by enhanced chemiluminescence (ECL, Amersham).

harvested by scraping into 10 mL of ice-cold PBS and pelleted by centrifugation at 200g for 1 min. The pellet was washed twice in 10 mL of ice-cold PBS and frozen at –70 °C. Adenylyl cyclase activity was measured in cell homogenates using a protein binding assay as previously described (30). Standard curve data were fitted to a logistic expression (GraphPad Software, San Diego, CA), and adenylyl cyclase activity was expressed as picomoles of cyclic AMP produced per minute per milligram of total protein (picomoles of cAMP per minute per milligram).

Whole-Cell cAMP Accumulation. Cells grown to ~90% confluency on 12-well plates were washed with PBS and stimulated at 37 °C with 250 μ L of PBS containing 300 μ M ascorbic acid, 1 mM isobutylmethylxanthine, and either vehicle (basal), 10 μ M Iso, or 100 μ M forskolin. Reactions were stopped after 0–45 min by placing the plates on ice, aspirating the medium, and adding 500 μ L of ice-cold ethanol. The contents of each well were collected, lyophilized, resuspended, and assayed for cAMP content by radioimmunoassay using [125 I]cAMP and anti-cAMP antibody as described previously (31).

Experimental Design and Statistics. Concentration–effect curves were analyzed by the iterative fitting program GraphPad Prism (GraphPad Software). Log concentration–effect curves were fitted to logistic expressions for single-site analysis. $t_{0.5}$ values for agonist-induced desensitization were obtained by fitting data to a single-exponential curve. Where appropriate, statistical significance was assessed by

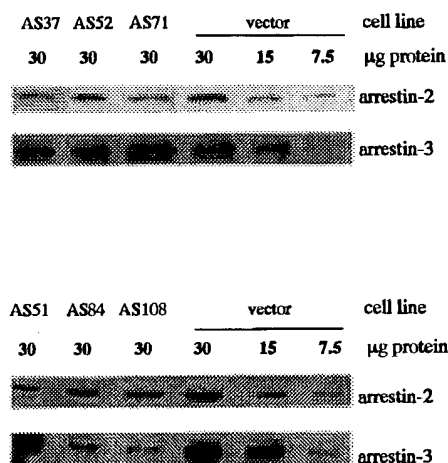


FIGURE 2: Analysis of arrestin levels in arrestin antisense mRNA expressing HEK293 cells. Whole cell lysates (7.5–30 μ g) were subjected to SDS–polyacrylamide gel electrophoresis followed by electrophoretic transfer and immunoblotting with rabbit polyclonal antibodies selective for arrestin-2 or arrestin-3. Six antisense mRNA-expressing cell lines (AS 37, 51, 52, 71, 84, and 108) (30 μ g of protein) were compared with vector-transfected HEK293-EBNA cells (30, 15, and 7.5 μ g of protein).

a Student's *t* test or by a two-way ANOVA using GraphPad Prism.

RESULTS

To test the effectiveness of different length arrestin-2 and arrestin-3 antisense constructs, COS-1 cells were cotransfected with wild-type arrestin-2 or -3 and one of eight different antisense constructs (panels A and B of Figure 1). These studies revealed that only the longer arrestin-2 and arrestin-3 antisense constructs (base pairs 1–801 to full length) caused a significant reduction in the level of arrestin expression.

Our initial attempts to express full-length antisense constructs stably integrated into the genome of HEK293 cells resulted in no reduction in endogenous arrestin levels (data not shown). To circumvent potential problems suggested by these results, we decided to express the antisense constructs in HEK293 cells that express the EBNA vector. The use of this vector helps to maintain plasmids such as pREP4 episomally and inhibits integration of the construct into the host cell genome. It was also decided that because use of the full-length antisense construct had proven to be unsuccessful, the shorter but still effective ~800 bp constructs would be utilized.

HEK293-EBNA cells were transfected with pREP4 vector alone, pREP4 vector containing the 801 bp arrestin-2 or arrestin-3 antisense constructs, or a combination of both antisense-containing constructs. After culture in medium containing hygromycin, surviving clones were isolated and expanded into clonal cell lines. Six clones that exhibited reductions in the arrestin-2 and/or arrestin-3 level were selected for further study. Of these, four had been transfected with pREP4 containing the arrestin-2 antisense construct (AS 37, 51, 52, and 71), one with pREP4 containing the arrestin-3 antisense construct (AS 84), and one that had been transfected with both antisense-containing constructs (AS 108) (Figure 2). The arrestin-2 antisense construct appeared to

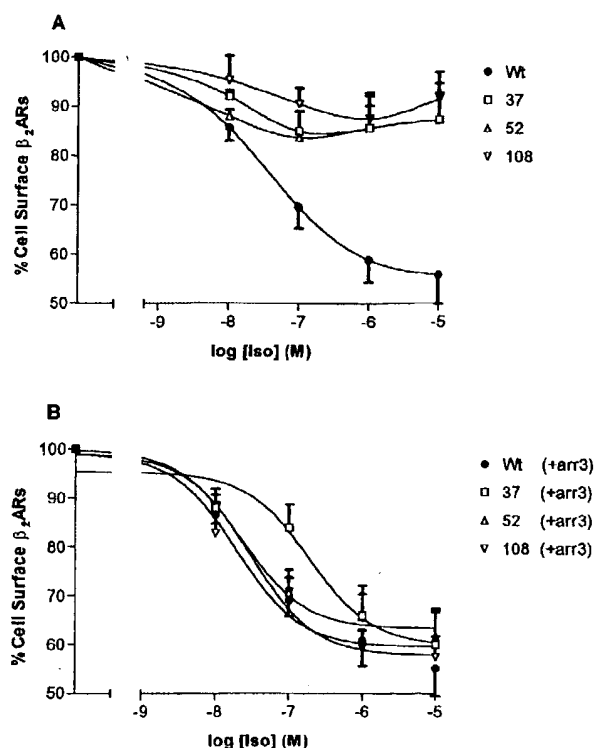


FIGURE 3: Effect of reduced arrestin levels on agonist-induced β_2 -AR internalization. Wild-type, AS 37, AS 52, and AS 108 cell lines were transiently transfected with 5 μ g of pcDNA3–Flag- β_2 AR alone (A) or together with 5 μ g of pcDNA3–arrestin-3 (+arr3) (B) and harvested ~48 h after transfection. Cells were incubated with 0–10 μ M (–)-isoproterenol at 37 °C for 20 min, washed, and then analyzed for cell surface β_2 ARs by ELISA as described in Experimental Procedures. β_2 AR expression levels were ~5 pmol/mg in all experiments. The data represent means \pm the standard error of four independent experiments.

selectively reduce only arrestin-2 levels, while the arrestin-3 construct reduced both arrestin-2 and -3 levels. Of the cell lines stably transfected with the arrestin-2 construct, all four exhibited a similar reduction (~50%) in arrestin-2 levels (Figure 2). By comparison, AS 84 and 108 exhibited an ~50% decrease in arrestin-2 and an ~75% decrease in arrestin-3 levels (Figure 2). Cells transfected with vector alone exhibited no change in endogenous arrestin levels compared to wild-type HEK293 cells, while endogenous GRK2 levels were not different among any of the clonal lines (data not shown).

To assess the biological effect of reduced arrestin levels, agonist- and time-dependent internalization of a transiently expressed Flag-tagged β_2 AR was initially investigated by ELISA analysis in each of the cell lines. In vector-transfected HEK293 cells, a dose-dependent loss of cell surface β_2 ARs was observed following a 20 min incubation with Iso (Figure 3A). This loss of cell surface β_2 ARs was not affected by overexpression of arrestin-3, confirming that arrestin-3 levels are not limiting in these cells (data not shown). Conversely, in clonal lines expressing various antisense constructs, agonist-induced internalization of the β_2 AR was almost completely abolished (Figure 3A). These results were confirmed by ligand binding analysis of cell surface β_2 ARs following a 30 min pretreatment with 10 μ M Iso (loss of cell surface receptors was 33.7 ± 1.8 , 7.3 ± 3.7 , and $7.0 \pm$

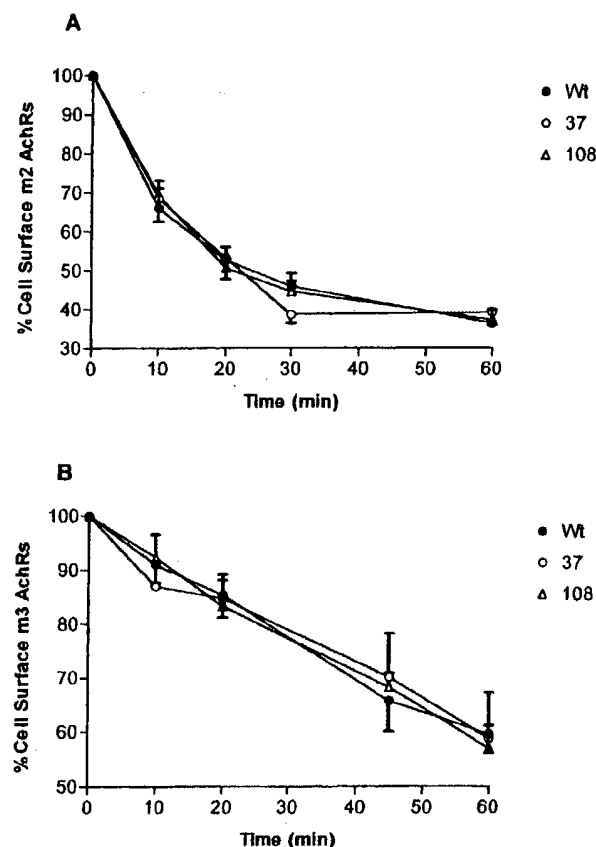


FIGURE 4: Effect of reduced arrestin levels on agonist-induced m2- and m3AChR internalization. HEK293 cells (AS 37, AS 108, and vector-transfected control), transiently transfected with 5 μ g of (A) pcDNA3-m2AChR or (B) pcDNA3-m3AChR, were harvested ~48 h after transfection. Cells were incubated with 1 mM carbachol at 37 °C for 0–60 min, washed extensively, resuspended, and then analyzed for cell surface m2- and m3AChRs as described in Experimental Procedures. m2AChR expression levels were ~1 pmol/mg in vector-transfected and antisense cells, while m3AChR levels were ~0.5 pmol/mg. Data represent means \pm the standard error of five independent experiments.

5.1% for vector-transfected, AS 37, and AS 108 cells, respectively; $n = 3$). Evidence that this effect was mediated by the selective reduction in cellular arrestin levels was provided by cell lines transiently overexpressing arrestin-3 (>10-fold increase in arrestin-3 levels compared to that in wild-type HEK293 cells) in which the attenuation of agonist-induced receptor internalization was reversed (Figure 3B). Similar results were obtained with AS 51, 71, and 84 cell lines (data not shown). Time course analysis of receptor internalization revealed that none of the lines with reduced arrestin levels demonstrated a significant level of β_2 AR internalization throughout a 45 min exposure to 10 μ M Iso, while significant internalization was observed in control cells (data not shown).

To demonstrate the selectivity of the arrestin antisense effects, m2- and m3AChRs were transiently overexpressed in antisense and control cells. Both of these receptors have been reported to be internalized by a non-arrestin-dependent mechanism (32, 33). Agonist-induced m2- and m3AChR internalization as assessed by changes in [3 H]NMS binding was unaltered in antisense-expressing and control cells (panels A and B of Figure 4). These results confirm that the

internalization of the m2- and m3AChRs is arrestin-independent in HEK293 cells.

We next examined the effects of reduced arrestin levels on endogenous β_2 ARs in HEK293 cells. These studies utilized the AS 37 and 108 clonal lines, which exhibited reductions in the level of only arrestin-2 or in both arrestin-2 and -3, respectively. The β_2 AR density was initially assessed in whole cells using the cell permeable ligand [125 I]iodopindolol. Displacement analysis in which the cell impermeable ligand CGP-12177 and cell permeable ligand alprenolol were utilized was used to assess surface and total β_2 AR density, respectively. Surface receptor levels were not different among the various lines (6.3 ± 1.2 , 6.4 ± 1.1 , and 6.8 ± 1.0 fmol of receptor/mg of protein for vector-transfected, AS 37, and AS 108 cells, respectively; $n = 4$). However, the total receptor density did appear to be somewhat higher in the antisense cell lines (8.2 ± 1.1 , 11.8 ± 1.9 , and 11.9 ± 1.5 fmol of receptor/mg of protein for vector-transfected, AS 37, and AS 108 cells, respectively; $n = 4$). Such results may suggest a role for arrestins in GPCR trafficking to the cell surface. However, since arrestins generally function by binding to agonist-occupied, GRK-phosphorylated GPCRs, we would not expect changes in cellular arrestin levels to directly affect constitutive internalization of the β_2 AR. Indeed, previous studies have demonstrated that even high-level overexpression of arrestins does not affect basal β_2 AR function (10).

Experiments were subsequently performed to identify potential differences in agonist-induced β_2 AR internalization among the clonal lines. Both AS 37 and AS 108 exhibited greatly reduced levels of agonist-dependent receptor internalization compared to control cells, although both lines exhibited significant surface receptor loss at higher agonist concentrations (panels A and B of Figure 5). However, the agonist concentration dependence of receptor internalization was not significantly altered in antisense cells (EC_{50} of 101 ± 9 nM for AS 37, 89 ± 8 nM for AS 108, 88 ± 8 nM for wild-type HEK293, and 86 ± 7 nM for vector-transfected cells). The time course of receptor internalization was also significantly slowed in antisense cells compared to control cells (panels C and D of Figure 5). Interestingly, although the $t_{0.5}$ of internalization was shorter in each antisense line ($t_{0.5}$ of 22.2 ± 4.8 and 13.8 ± 3.7 min for AS 37 and 108, respectively, compared to 6.7 ± 1.9 and 5.3 ± 2.1 min for wild-type and vector-transfected HEK293 cells, respectively), both antisense lines exhibited significant levels of receptor internalization at later time points.

Since arrestins have also been implicated in the uncoupling of receptor and G protein (2, 3, 8, 10–12), we next examined the effect of reduced arrestin levels on the desensitization of endogenous β_2 ARs. Agonist-stimulated adenylyl cyclase activity was first assessed in homogenates from wild-type, vector-transfected, and antisense-expressing HEK293 cells. There were no significant differences among the various cell lines in basal (untreated) or Iso (0.01–10 μ M)-, NaF (10 mM)-, or forskolin (10 μ M)-stimulated adenylyl cyclase activities (data not shown). The time course and concentration dependence of agonist-mediated desensitization were then examined by pretreating cells with Iso (0.1–10 μ M) for 0–45 min prior to homogenization of the cells and subsequent determination of adenylyl cyclase activity (Figure 6). The rate of β_2 AR desensitization was significantly slower

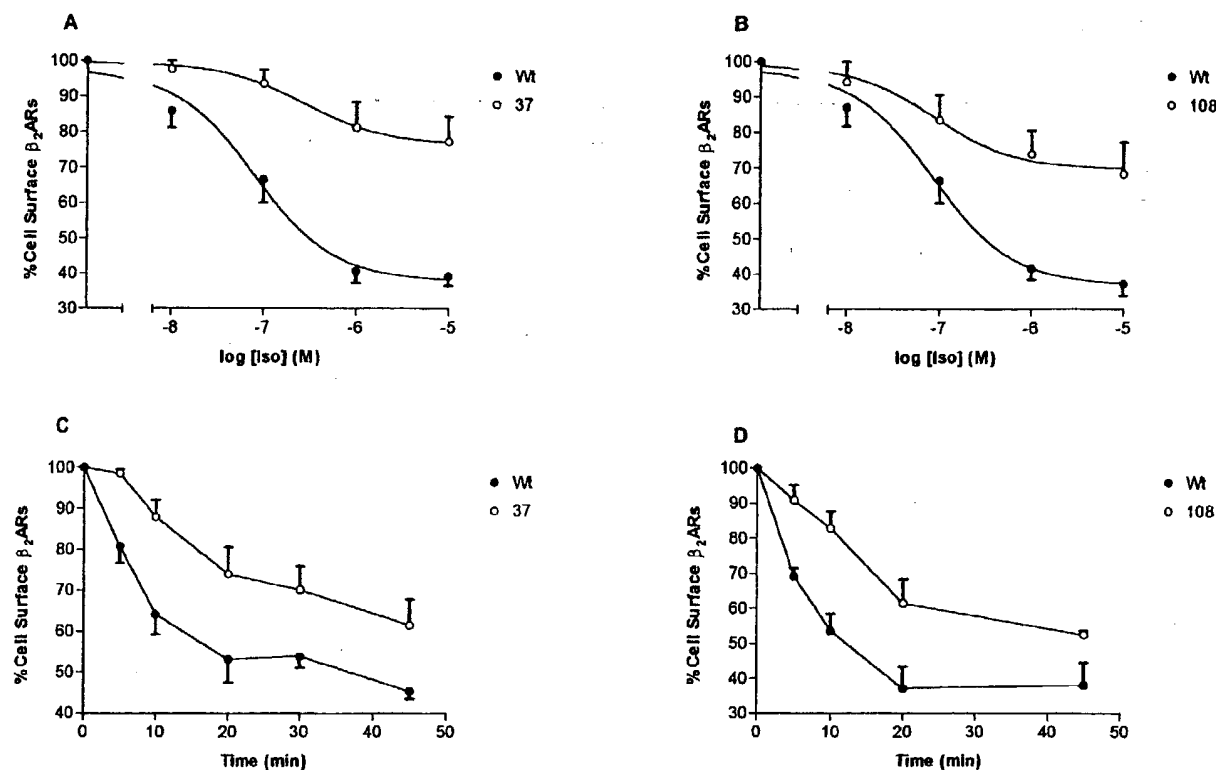


FIGURE 5: Effect of a reduced level of arrestin expression on endogenous β_2 AR internalization. (A and B) Cells were treated with a range of concentrations (0.01–10 μ M) of (–)-isoproterenol for 20 min and washed extensively in PBS, and the cell surface β_2 AR level was analyzed as described in Experimental Procedures. (C and D) Cells were treated with 10 μ M (–)-isoproterenol for 0–45 min and washed extensively in PBS, and the surface β_2 AR level was analyzed. Data represent means \pm the standard error of five independent experiments. Note that the total number of receptors was unaffected by agonist pretreatment (8.4 ± 1.6 , 12.5 ± 1.9 , and 12.7 ± 1.5 fmol of receptor/mg of protein prior to agonist treatment vs 8.0 ± 1.5 , 11.9 ± 1.3 , and 11.8 ± 1.3 fmol/mg following a 45 min treatment with 10 μ M Iso for vector-transfected, AS 37, and AS 108 cells, respectively).

in both antisense cell lines ($t_{0.5}$ of 5.0 ± 0.7 and 8.4 ± 0.8 min for AS 37 and 108, respectively, compared to 2.6 ± 0.4 and 1.3 ± 0.5 min for wild-type and vector-transfected cells, respectively) (panels A and B of Figure 6). Additional analysis of these curves revealed that the initial rapid loss of adenylyl cyclase activity (during the first 10 min) was significantly faster in control cells than in antisense-expressing cells (3.2 ± 0.5 and $4.3 \pm 0.4\%$ loss of activity per minute for wild-type and vector-transfected cells, respectively vs 1.9 ± 0.3 and $1.4 \pm 0.3\%$ for AS 37 and 108 cell lines, respectively). In contrast, the loss of adenylyl cyclase activity during the latter phase (10–45 min) was somewhat greater in antisense-expressing cells (0.11 ± 0.06 and $0.10 \pm 0.07\%$ loss per minute for wild-type and vector-transfected cells, respectively, vs 0.24 ± 0.10 and $0.29 \pm 0.09\%$ for AS 37 and 108 cell lines, respectively). This is likely due to the increased number of non-desensitized receptors available in antisense cells during this latter period that can undergo agonist-induced desensitization. The extent of receptor desensitization was also greatly reduced over a range of agonist concentrations in antisense versus control cells, although the EC_{50} of desensitization was not significantly changed (EC_{50} values of 10.8 ± 4.2 and 21.0 ± 7.8 nM for AS 37 and 108, respectively, compared to values of 18.2 ± 4.2 and 11.4 ± 5.2 nM for wild-type and vector-transfected cells, respectively) (panels C and D of Figure 6).

β_2 AR desensitization was also assessed in intact cells by measuring a time course of cAMP production following

addition of agonist. The level of β_2 AR-stimulated cAMP accumulation was significantly greater in antisense than in control cells, with a 2–3-fold increase in the level of cAMP production following a 45 min incubation with Iso (Figure 7A). In contrast, stimulation with 100 μ M forskolin produced equivalent increases in the cAMP level among the various cell lines (Figure 7B).

Previous studies have shown that β_2 AR internalization (which is mediated by arrestins) is critical to the process of receptor resensitization (14–16). To test this possibility, vector-transfected, AS 37, and AS 108 cell lines were treated with Iso for 30 min, then washed, and allowed to resensitize in fresh medium. As shown in Figure 8, β_2 AR resensitization was greatly impaired in cells with reduced arrestin levels compared to that exhibited in control cells.

The ability of reduced arrestin levels to effectively attenuate β_2 AR regulation prompted us to also characterize the regulation of additional endogenous GPCRs in these cells. In this regard, we studied the desensitizations of two GPCRs that are also coupled to activation of adenylyl cyclase in these cells, the A_{2b} adenosine receptor (34) and a prostaglandin E_2 (PGE₂)-stimulated receptor. In wild-type cells (and vector-transfected cells), pretreatment with the adenosine receptor agonist NECA resulted in rapid desensitization that reached a level of $\sim 75\%$ after 30 min (Figure 9A). Similarly, the PGE₂ response was also subject to rapid desensitization reaching $\sim 75\%$ following a 20 min pretreatment with agonist (Figure 9B). Interestingly, both the NECA- and PGE₂-

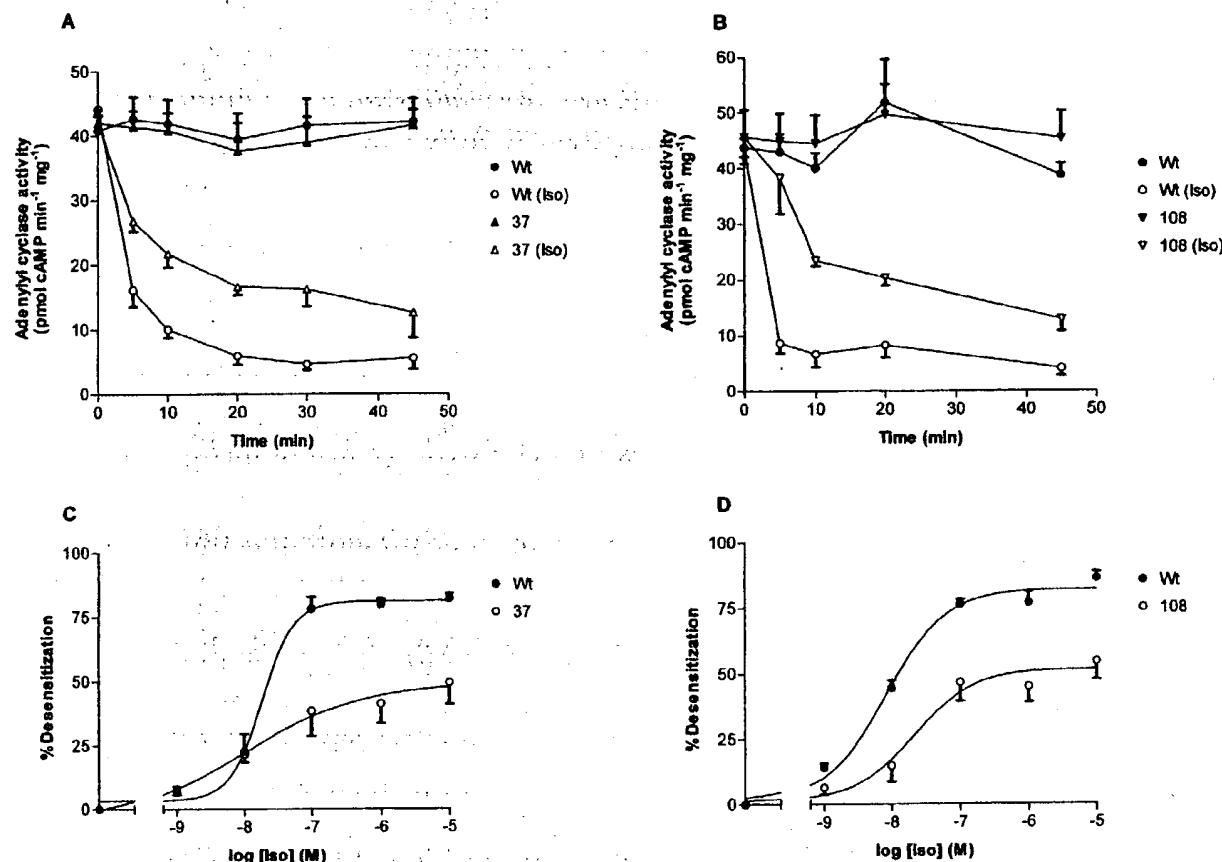


FIGURE 6: Effect of a reduced level of arrestin expression on agonist-induced desensitization of endogenous β_2 ARs. (A and B) Cells were pretreated with either vehicle or (–)-isoproterenol (10 μ M) for 0–45 min. Cells were harvested, and isoproterenol (10 μ M)-stimulated adenylyl cyclase activity was measured in homogenates as described in Experimental Procedures. (C and D) Cells were pretreated with either vehicle or (–)-isoproterenol (0.01–10 μ M) for 20 min. Cells were harvested, and isoproterenol (10 μ M)-stimulated adenylyl cyclase activity was measured in homogenates as described above. Data represent means \pm the standard error of five independent experiments. The level of desensitization was significantly lower for β_2 AR in AS 37 than in wild-type HEK293 cells and in AS 108 than in vector-transfected control HEK293 cells ($p < 0.05$, two-way ANOVA).

promoted desensitization were significantly attenuated in the AS 37 and AS 108 lines (Figure 9). Thus, endogenous arrestins appear to play an important role in regulating the rapid agonist-promoted desensitization of the A_{2b} adenosine receptor and a PGE_2 -stimulated receptor in HEK293 cells.

DISCUSSION

This study is the first to demonstrate that expression of antisense mRNAs can effectively reduce endogenous cellular arrestin levels. Using this technique, we have characterized the effects on the regulation of both exogenously expressed (β_2 AR, m2AChR, and m3AChR) and endogenous (β_2 AR, A_{2b} adenosine, and PGE_2 -stimulated) GPCRs induced by significant reductions in arrestin levels.

Initial characterization of the effectiveness of different length antisense mRNAs in reducing the level of arrestin overexpression in COS-1 cells yielded candidate constructs for arrestin-2 and arrestin-3. HEK293-EBNA cells were then utilized to stably transfect and maintain the transfection vector (pREP4) and incorporated antisense construct episomally. This approach seemed to optimize the effectiveness of the antisense signal, since our initial studies in which the antisense construct was integrated into the host cell genome

proved to be unsuccessful. Interestingly, although the arrestin-2 construct selectively reduced arrestin-2 levels, the arrestin-3 antisense construct reduced the level of expression of both arrestin-2 and -3. The lack of specificity of our arrestin-3 antisense construct might be attributed to regions of high homology between the corresponding human arrestin-2 and arrestin-3 mRNAs (35, 36). However, in this regard it is unclear why the arrestin-2 antisense mRNA did not alter arrestin-3 expression. Future studies using regions of lower homology between arrestin-2 and -3 may help identify antisense sequences with greater specificity, although our ~100–500 bp constructs proved to be ineffective.

Our initial studies focused on the internalization of an overexpressed Flag-tagged β_2 AR. These studies showed that reductions in the level of endogenous arrestin expression in antisense-expressing cells effectively reduced the level of receptor internalization whether assessed by cell surface immunoreactivity or ligand binding. Interestingly, this abolition of receptor internalization was evident in all antisense cell lines, even those with only a 50% reduction in the arrestin-2 level alone (AS 37, 51, 52, and 71). To confirm that reduced arrestin expression and not nonspecific effects were mediating the lack of receptor internalization, receptors were transiently coexpressed with arrestin-3 in an attempt

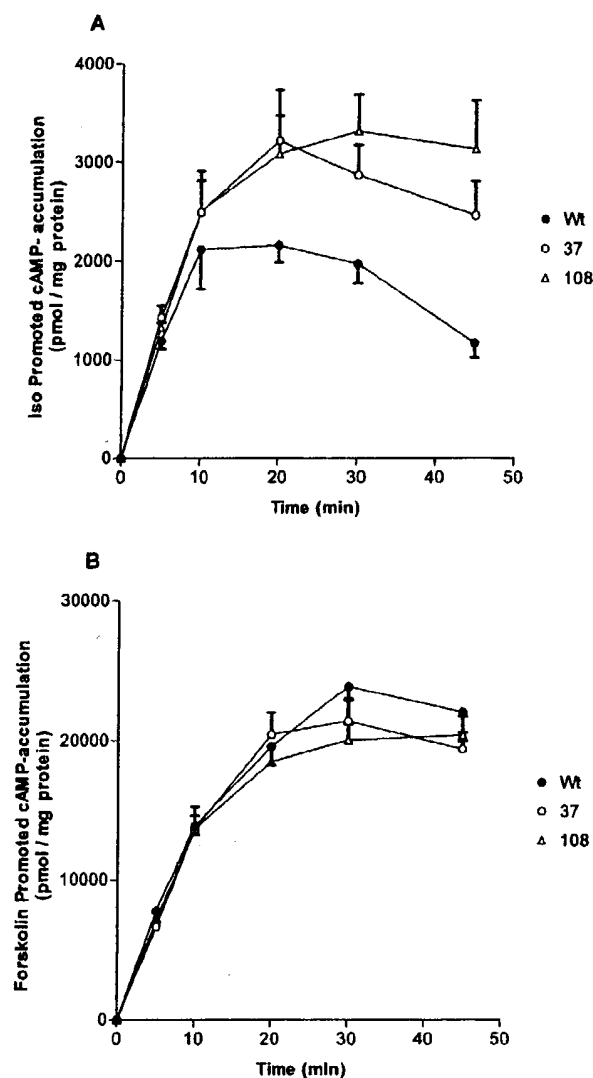


FIGURE 7: Cyclic AMP accumulation in intact wild-type and antisense-expressing HEK293 cells. Cyclic AMP levels were measured in vector-transfected (Wt), AS 37, and AS 108 cells after the addition of 10 μ M (—) isoproterenol (A) or 100 μ M forskolin (B). Values represent means \pm the standard error from four independent experiments. The level of Iso-stimulated cAMP accumulation was significantly greater in AS 37 and 108 cells than in wild-type cells ($p < 0.05$, two-way ANOVA).

to restore internalization. Overexpression of arrestin-3 (~10-fold over basal) had a minimal effect on β_2 AR internalization in wild-type and vector control cells, indicating that endogenous arrestin levels are sufficient to mediate receptor internalization even when the level of receptor expression is increased, as previously reported (18, 21). In antisense-expressing cells, overexpression of arrestin-3 was sufficient to restore receptor internalization to control levels. This finding illustrates that the selective reduction in arrestin levels mediated the attenuation of receptor internalization. To further explore the specificity of the arrestin antisense effects, we examined internalization of the m2- and m3AChR in the AS 37 and 108 lines. The agonist-dependent internalization of the m2- and m3AChRs was unaffected by reductions in arrestin levels, confirming previous studies that demonstrated that internalization of these receptors in HEK293 cells was

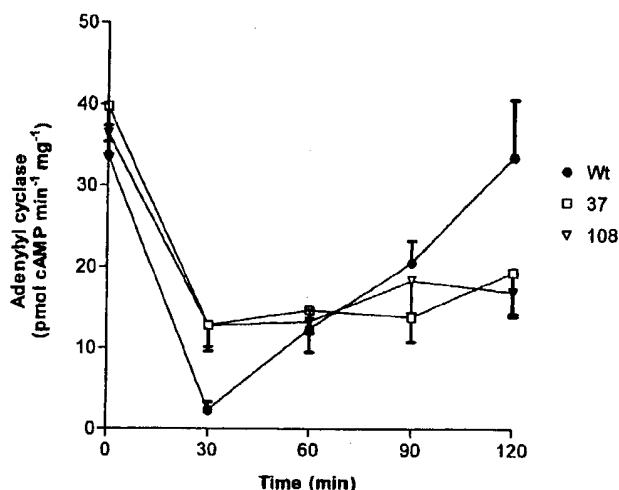


FIGURE 8: Effect of a reduced level of arrestin expression on β_2 -AR resensitization. Vector-transfected (Wt) and antisense cells (AS 37 and 108) were pretreated with 10 μ M (—) isoproterenol for 30 min, washed, and then reincubated with fresh medium for an additional 90 min. Cells were harvested before treatment and 0, 30, 60, and 90 min after agonist washout and assayed for isoproterenol-stimulated adenylyl cyclase activity as described in Experimental Procedures. Values represent means \pm the standard error from four independent experiments.

arrestin-independent (32, 33). These results indicate that antisense arrestin mRNA expression specifically affected those mechanisms mediating β_2 AR internalization while not affecting arrestin-independent mechanisms mediating internalization of m2- and m3AChR.

Subsequent studies focused on the regulation of endogenous, natively expressed β_2 ARs. The level of agonist-stimulated receptor internalization was reduced in both AS 37 and 108 cell lines. The time course of Iso-stimulated internalization revealed that reduced arrestin levels inhibited rather than stopped internalization. This suggests either that the remaining endogenous arrestins mediated receptor internalization or that mechanisms other than arrestin-mediated internalization are involved. For example, β_2 ARs have been shown to colocalize with caveolae (37), and this may represent an arrestin-independent mechanism of internalization similar to that suggested for the bradykinin receptor (38).

The reduction in the extent of receptor internalization caused by arrestin antisense expression was more dramatic with overexpressed than with endogenous β_2 AR. This may indicate that a threshold for measurable receptor internalization levels exists that is dependent on both receptor and arrestin expression. For example, in a system with a greatly increased level of receptor expression and a reduced level of arrestin expression, receptor internalization may no longer be evident. Conversely, when the level of receptor expression is low, extant endogenous arrestins in the antisense-expressing cells can still mediate a measurable level of receptor internalization, albeit at a slower rate.

We observed little difference in endogenous β_2 AR internalization between AS 37 and 108 lines even though the latter exhibits a much greater reduction in arrestin-3 levels. This finding suggests that arrestin-3 plays a relatively lesser role in endogenous β_2 AR internalization or that the remaining endogenous arrestins are sufficient to induce receptor internalization. No clear functional differences have yet to

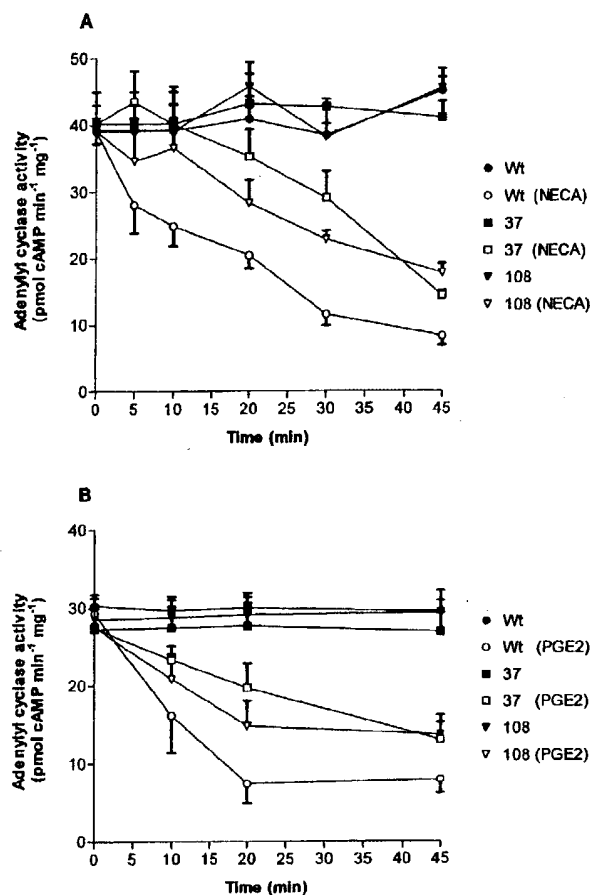


FIGURE 9: Effect of a reduced level of arrestin expression on agonist-induced desensitization of endogenous A_{2b} adenosine receptor (A) and PGE₂-stimulated adenylyl cyclase activity (B). Cells were pretreated with either vehicle or (A) NECA (100 μ M) or (B) PGE₂ (1 μ M) for 0–45 min. Cells were harvested, and (A) NECA (100 μ M)- or (B) PGE₂ (1 μ M)-stimulated adenylyl cyclase activity was measured in homogenates as described in Experimental Procedures. Data represent means \pm the standard error of five independent experiments. The level of desensitization was significantly lower for A_{2b} adenosine receptor and PGE₂-stimulated adenylyl cyclase activity in AS 37 and AS 108 than in vector-transfected control HEK293 cells ($p < 0.05$, two-way ANOVA).

be identified between arrestin-2 and arrestin-3 (13). Identification of an arrestin-3 antisense construct that specifically reduces endogenous arrestin-3 protein may help to resolve such differences, if any exist. It will also be interesting to investigate if other methods of reducing arrestin function used in conjunction with reduced arrestin expression can further alter receptor signaling.

The role of arrestins in the agonist-induced desensitization of a number of GPCRs has previously been reported (13). In intact cells, agonist-specific desensitization of the β_2 AR was significantly attenuated by expression of antisense constructs. This indicated, as previously suggested, that arrestins are involved in the desensitization of β_2 AR responses (10). Importantly, cAMP accumulation as a result of forskolin stimulation was identical in antisense and control cells, suggesting the lack of involvement of arrestins in this response. Similarly, β_2 AR desensitization assessed in cell homogenates was also attenuated by arrestin antisense constructs. Arrestin antisense expression increased the $t_{0.5}$

of β_2 AR desensitization, although a similar level of desensitization was ultimately achieved at later time points. This could indicate that remaining endogenous arrestins interact with and uncouple phosphorylated receptors from G protein or that mechanisms other than arrestin-mediated uncoupling are involved.

Arrestin-2 has been shown to play an integral role in regulating not only desensitization and intracellular trafficking of GPCRs but also the ability of the β_2 AR to resensitize (39). These findings were further confirmed in our study. In antisense-expressing cells, no significant resensitization of response was observed, indicating that arrestins play a major role in receptor resensitization.

Since reduced arrestin levels effectively attenuated β_2 AR regulation, we next used the antisense lines to characterize the involvement of arrestins in desensitization of the endogenously expressed A_{2b} adenosine receptor (34). While GRK2 has previously been implicated in the desensitization of A_{2b} adenosine receptors in NG108-15 cells (30), a role for arrestins in this process has not been characterized. Agonist-mediated desensitization of the A_{2b} adenosine receptor was significantly attenuated in cells containing reduced arrestin levels, implicating arrestin involvement in the desensitization of A_{2b} adenosine receptors in HEK293 cells.

We also used the antisense lines to characterize desensitization of a PGE₂-stimulated receptor in these cells. At present, it is unclear which prostanoid receptors are endogenously expressed in HEK293 cells, although the rapid desensitization of the PGE₂ response we observed mirrors that of the prostaglandin EP₄ receptor (40). Interestingly, desensitization of the PGE₂-stimulated adenylyl response was also significantly attenuated in antisense-expressing cells. This provides the first evidence of arrestin involvement in the desensitization of prostanoid receptors.

In summary, this study shows that an antisense strategy can successfully be employed to reduce endogenous arrestin levels and effect changes in β_2 AR internalization, desensitization, and resensitization. Further, our results demonstrate that the antisense cells can also be effectively used to examine arrestin involvement in the regulation of other GPCRs. Future studies will attempt to investigate the regulation of additional endogenous and transfected GPCRs in these cells.

ACKNOWLEDGMENT

We thank R. Penn for critical reading of the manuscript and members of the Benovic lab for helpful discussions. Human cDNAs for arrestin-2 and arrestin-3 were generously provided by A. De Blasi (Istituto di Ricerche Farmacologiche Mario Negri, Santa Maria Imbaro, Italy) and B. Rapoport (University of California, San Francisco, CA), respectively. m2 and m3AChR cDNAs were kindly provided by E. Peraulta (Harvard University, Cambridge, MA), while the anti-cAMP antibody was a gift from M. Ascoli (University of Iowa, Iowa City, IA).

REFERENCES

1. Penn, R. B., and Benovic, J. L. (1998) in *Handbook of Physiology* (Conn, P. M., Ed.) Vol. 1, pp 125–164, Oxford University Press, New York.
2. Kuhn, H., Hall, S. W., and Wilden, U. (1984) *FEBS Lett.* 176, 473–478.

3. Krupnick, J. G., Gurevich, V. V., and Benovic, J. L. (1997) *J. Biol. Chem.* 272, 18125–18131.
4. Palczewski, K., and Smith, W. C. (1996) *Exp. Eye Res.* 63, 599–602.
5. Sakuma, H., Inana, G., Murakami, A., Higashide, T., and McLaren, M. J. (1996) *FEBS Lett.* 382, 105–110.
6. Craft, C. M., Whitmore, D. H., and Wiechmann, A. F. (1994) *J. Biol. Chem.* 269, 4613–4619.
7. Lohse, M. J., Benovic, J. L., Codina, J., Caron, M. G., and Lefkowitz, R. J. (1990) *Science* 248, 1547–1550.
8. Altamadal, H., Ariza, J. L., Aoki, C., Dawson, T. M., Codina, J., Kwatra, M. M., Synder, S. H., Caron, M. G., and Lefkowitz, R. J. (1992) *J. Biol. Chem.* 267, 17882–17890.
9. Sterne-Marr, R., Gurevich, V. V., Goldsmith, P., Bodine, R. C., Sanders, C., Donoso, L. A., and Benovic, J. L. (1993) *J. Biol. Chem.* 268, 15640–15648.
10. Pippig, S., Andexinger, S., Daniel, K., Puzicha, M., Caron, M. G., Lefkowitz, R. J., and Lohse, M. J. (1993) *J. Biol. Chem.* 268, 3201–3208.
11. Freedman, N. J., Liggett, S. B., Drachman, D. E., Pei, G., Caron, M. G., and Lefkowitz, R. J. (1995) *J. Biol. Chem.* 270, 17953–17961.
12. Diviani, D., Lattion, A. L., Larbi, N., Kunapuli, P., Pronin, A., Benovic, J. L., and Cotecchia, S. (1996) *J. Biol. Chem.* 271, 5049–5058.
13. Krupnick, J. G., and Benovic, J. L. (1998) *Annu. Rev. Pharmacol. Toxicol.* 38, 289–319.
14. Yu, S. S., and Lefkowitz, R. J. (1993) *J. Biol. Chem.* 268, 337–341.
15. Pippig, S., Andexinger, S., and Lohse, M. J. (1995) *Mol. Pharmacol.* 47, 666–676.
16. Krueger, K. M., Daaka, Y., Pitcher, J. A., and Lefkowitz, R. J. (1997) *J. Biol. Chem.* 272, 5–8.
17. Bogatkevitch, G. S., Lenz, W., Jakobs, K. H., and Van Koppen, C. J. (1996) *Mol. Pharmacol.* 50, 424–429.
18. Mundell, S. J., and Kelly, E. (1998) *Br. J. Pharmacol.* 125 (7), 1594–1600.
19. Ferguson, S. S. G., Downey, W. E. I., Colapietro, A.-M., Barak, L. S., Menard, L., and Caron, M. G. (1996) *Science* 271, 363–365.
20. Goodman, O. B., Jr., Krupnick, J. G., Santini, F., Gurevich, V. V., Penn, R. B., Gagnon, A. W., Keen, J. H., and Benovic, J. L. (1996) *Nature* 383, 447–450.
21. Krupnick, J. G., Goodman, O. B., Jr., Keen, J. H., and Benovic, J. L. (1997) *J. Biol. Chem.* 272, 15011–15016.
22. Menard, L., Ferguson, S. S. G., Zhang, J., Lin, F. T., Lefkowitz, R. J., Caron, M. G., and Barak, L. S. (1997) *Mol. Pharmacol.* 51, 800–808.
23. Krupnick, J. G., Santini, F., Gagnon, A. W., Keen, J. H., and Benovic, J. L. (1997) *J. Biol. Chem.* 272, 32507–32512.
24. Orsini, M. J., and Benovic, J. L. (1998) *J. Biol. Chem.* 273, 34616–34622.
25. Rohrer, D. K., and Kobilka, B. K. (1998) *Physiol. Rev.* 78, 35–52.
26. Kalkbrenner, F., Dippel, E., Wittig, B., and Schultz, G. (1996) *Biochim. Biophys. Acta* 1314, 125–139.
27. Shih, M., and Malbon, C. C. (1994) *Proc. Natl. Acad. Sci. U.S.A.* 91 (25), 12193–12197.
28. Laemmli, U. K. (1970) *Nature* 227, 680–685.
29. Daunt, D. A., Hurt, C., Hein, L., Kallio, J., Feng, F., and Kobilka, B. K. (1997) *Mol. Pharmacol.* 51, 711–720.
30. Mundell, S. J., Benovic, J. L., and Kelly, E. (1997) *Mol. Pharmacol.* 51, 991–998.
31. Penn, R. B., Kelsen, S. G., and Benovic, J. L. (1994) *Am. J. Respir. Cell Mol. Biol.* 11, 496–505.
32. Pals-Rylaarsdam, R., Gurevich, V. V., Lee, K. B., Ptasienski, J. A., Benovic, J. L., and Hosey, M. M. (1997) *J. Biol. Chem.* 272, 23682–23689.
33. Lee, K. B., Pals-Rylaarsdam, R., Benovic, J. L., and Hosey, M. M. (1998) *J. Biol. Chem.* 273, 12967–12972.
34. Cooper, J., Hill, S. J., and Alexander, S. P. H. (1997) *Br. J. Pharmacol.* 122, 546–550.
35. Parrauti, G., Peracchia, F., Sallese, M., Ambrosini, G., Masini, M., Rotilio, D., and De Blasi, A. (1993) *J. Biol. Chem.* 268, 9753–9761.
36. Rapoport, B., Kaufman, K. D., and Chazenbalk, G. D. (1992) *Mol. Cell. Endocrinol.* 84, R39–R43.
37. Dupree, P., Parton, R. G., Raposo, G., Kurzchalia, T. V., and Simons, K. (1993) *EMBO J.* 12, 1597–1605.
38. de Weerd, W. F., and Leeb-Lundberg, L. M. (1997) *J. Biol. Chem.* 272, 17858–17866.
39. Zhang, J., Barak, L. S., Winkler, K. E., Caron, M. G., and Ferguson, S. S. G. (1997) *J. Biol. Chem.* 272, 27005–27014.
40. Nishigaki, N., Negishi, M., and Ichikawa, A. (1996) *Mol. Pharmacol.* 50, 1031–1037.

B1990361V

Review

GTP-binding–protein-coupled receptor kinases

Two mechanistic models

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(Received 21 March/29 May 1997) – EJB 97 0412/0

Six vertebrate protein kinases (G-protein-coupled receptor kinases; GRKs) that regulate the function of G-protein-coupled receptors (GPCRs) were recently cloned; several distinct properties set them apart from conventional second-messenger regulated protein kinases. It appears that GRKs bind GPCR* through two separate sites: a high-affinity site, which involves intracellular loops of the activated receptor, and the lower-affinity site, encompassing the phosphorylation region. The high-affinity interaction may involve complementary structural elements of GRKs and GPCRs* rather than precise amino acid alignment, thus allowing broad and overlapping specificities of these kinases, in spite of differences in the sequences of GPCRs. In addition, GRK structures are modified by several posttranslational modifications, including phosphorylation, autophosphorylation, prenylation, carboxymethylation, and palmitoylation, probably affecting properties of these enzymes. While GRKs phosphorylate and inactivate receptor molecules which are engaged in G-protein activation, controversy surrounds whether GRKs might be activated and phosphorylate unstimulated GPCRs, leading to a desensitization of a larger population of the receptors. In this review, mechanistic aspects of GPCR* phosphorylation related to the distinct properties, regulation and modes of action of GRKs are described.

Keywords: G-protein-coupled receptor; rhodopsin kinase; rhodopsin; arrestin; signal transduction; G-protein-coupled receptor kinase.

For 40 years, protein phosphorylation has been recognized as a fundamental mechanism of cellular regulation, affecting enzymatic activities, cytoskeletal organization and signaling by receptors, including the G-protein-coupled receptors (GPCRs). Phosphorylation of GPCRs after agonist stimulation results in shortened physiological responses and desensitization of signal transduction pathways in which these receptors participate (Fig. 1). While GPCRs must be turned off to prevent continuing activation of the entire population of G-protein, their phosphorylation and subsequent quenching must be timed properly in order for some G-protein molecules to become activated by GPCR*. Quenching and desensitization of GPCRs by phosphorylation are well established phenomena that involve two types of Ser/Thr protein kinases: G-protein receptor kinases (GRKs; EC.2.7.1.—) and kinases activated by second-messengers (e.g.

protein kinase A or protein kinase C). Several comprehensive reviews concerning different aspects of receptor phosphorylation have been published (Palczewski and Benovic, 1991; Inglese et al., 1993; Haga et al., 1994b; Premont et al., 1995; Lohse et al., 1996; Ferguson et al., 1996b; Chuang et al., 1996b; Böhm et al., 1997; Stoffel et al., 1997).

GPCRs exist in an inactive conformation, which upon agonist binding convert to an active form (GPCR*; Fig. 1). The activation persists for a short time allowing GPCR* to catalyze nucleotide exchange (from GDP to GTP) on a fraction of G-protein molecules (activation). The amplification gain and the type of G-protein which is activated depends on the specificity of GPCR and the concentration of the agonist. For example, the gain for a single photolyzed rhodopsin molecule is several hundred G_i (transducin) molecules. GRK(s) also binds to cytoplasmic loops of GPCR* forming a stable complex. The activated receptors are then phosphorylated at the C-terminus (rhodopsin, the β_2 -adrenergic receptor) or the third cytoplasmic loop (the m2-muscarinic receptor). Phosphorylation has only a small inhibitory effect on G-protein activation, but it is an essential step for GPCR* desensitization (Chen et al., 1995b; Yamamoto et al., 1997). *In vitro*, GPCR* can be phosphorylated to a high stoichiometry of 7–9 phosphate groups/GPCR molecule. However, a single phosphate/receptor molecule, as observed *in vivo* (see also Fig. 3), is sufficient for quenching signal transduction (Bennett and Sitaramayya, 1988). The affinity of the kinase for the activated receptor is weakened by autophosphorylation or other mechanisms, such as dissociation of G-protein $\beta\gamma$ -dimers from GRK2 or GRK3 and re-association with α -subunits of G-

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Abbreviations. GRK, G protein-coupled receptor kinase; GPCR, G-protein-coupled receptor; GPCR*, the active form of G protein-coupled receptor; Arr, arrestin; PrP2A, protein phosphatase 2A; PtdIns(4,5) P_2 , phosphatidylinositol 4,5-bisphosphate.

Enzymes. G-protein-coupled receptor kinase GRK1, rhodopsin kinase, (EC 2.7.1.125); G protein-coupled receptor kinases GRK2 and GRK3, β -adrenergic receptor kinases 1 and 2 (EC 2.7.1.126); G-protein-coupled receptor kinases GRK4, GRK5 and GRK6 (EC 2.7.1.—).

Note. This Review will be reprinted in *EJB Reviews* 1997 which will be available in April 1998.

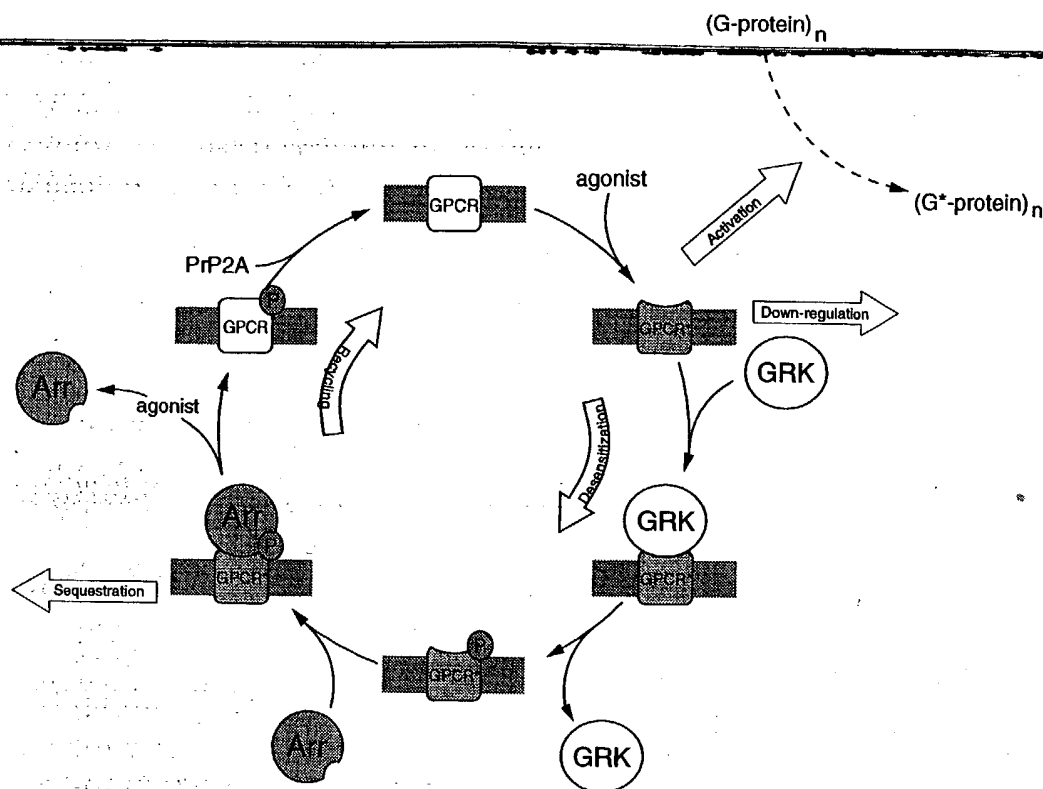


Fig. 1. The quenching and desensitization cycle of GPCRs. Upon stimulation by agonist, GPCR undergoes several physiologically important processes, including activation of G-protein, desensitization, sequestration and regeneration (recycling). See the text for details.

protein molecules, and the kinase is replaced by the regulatory protein, arrestin (Arr). In the GPCR*·P_i·Arr complex, GPCR* is unable to interact with the G-protein. Accumulation of such complexes, which may persist for a long time, would prevent the receptor from participating in another round of activation. Alternatively, the long-term desensitization in response to prolonged agonist-stimulation may also lead to reduced expression and to increased rate of receptor degradation (down-regulation) (Ferguson et al., 1996b).

The GPCR*·P_i·Arr complex is sequestered in endosomal vesicles, effectively lowering their densities on the plasma membrane (Tsuga et al., 1994). The sequestration (internalization) process is largely uncharacterized at the biochemical level, but likely involves the complex GPCR*·P_i·Arr (Ferguson et al., 1996a,b; Zhang et al., 1996; Goodman et al., 1996). GRK2 appears also to colocalize with the receptor during internalization (Ruiz-Gomez and Mayor, 1997). For photolyzed rhodopsin, removal of the agonist by reduction of all-*trans*-retinal to its alcohol leads to the release of Arr and dephosphorylation of the receptor by a membrane-associated form of protein phosphatase 2A (PrP2A) (Palczewski et al., 1989b; Fowles et al., 1989). Rhodopsin is fully regenerated when a new 11-*cis*-retinal chromophore combines with opsin. For other GPCR*·P_i·Arr complexes which are sequestered, lowering the pH in intracellular organelles may lead to conformational changes of the receptor and the activation of a latent form of PrP2A and dephosphorylation (Pitcher et al., 1995b; Krueger et al., 1997) (recycling, regeneration).

Vertebrate Arr are encoded by four genes, which give rise to several mRNA forms by alternative splicing (Palczewski, 1994). Which GRK and Arr participate in the inactivation of a particular GPCR* depends on their localization and kinetic properties.

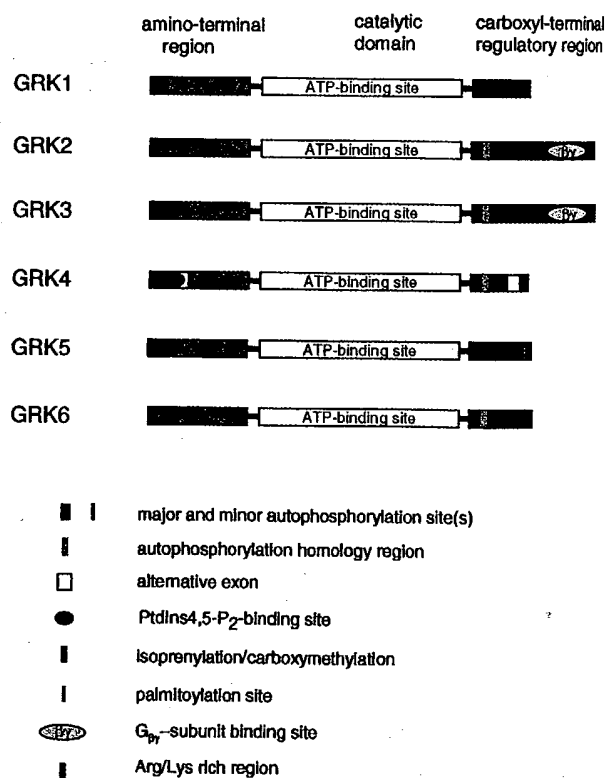


Fig. 2. Schematic representation of the domain structure of GRKs. GRKs are monomeric enzymes that contain the catalytic domain in the middle of the sequence. N- and C-terminal regions are sites of post-translational modifications and are regulatory regions of the kinases.

Table 1. G-protein-coupled receptor kinases (GRKs). Posttranslational modifications were not considered in the molecular mass and number of amino acids. For most GRKs, the cell type expression is unknown. The references listed are recent review articles or papers related to the enzymological characterization of GRKs.

Kinase	Molecular mass	Number of amino acids	Gene structure	Chromosomal localization (human)	Tissue/cellular expression	Co- and post-translational modifications	<i>In vitro</i> substrates and agonist-stimulated receptors	Comments	References	EMBL/GenBank accession no.
GRK1 (rhodopsin kinase)	62934 Da	561	7 exons	13q34	retinal photoreceptors	prenylated and carboxyl-methylated at Cys558; autophosphorylated at Ser21, Ser489, and Thr489	photolyzed rhodopsin; constitutively active mutants of opsin, opsin with all- <i>trans</i> -retinal, β 1-, β 2-adrenergic receptors, acidic peptides and proteins	the most specialized of all GRKs	Zhao et al. (1995)	P28327
GRK2 (β -adrenergic receptor kinase 1)	79646	689	21 exons	11q13	ubiquitous	unknown; phosphorylated and activated by protein kinase C	α 1-, β 2-, β 1-, β 2-adrenergic receptors, thrombin receptor, substance-P receptor, olfactory receptor, photolyzed rhodopsin, <i>N</i> -formyl peptide receptor, m3-muscarinic receptor	activated by lipids, and β -subunits of G-proteins	Benovic et al. (1987)	P2146
GRK3 (β -adrenergic receptor kinase 2)	79803	688	—	22q11	ubiquitous	unknown	α 1-, α 2-, β 1-, β 2-adrenergic receptors, thrombin receptor, substance-P receptor, olfactory receptor, photolyzed rhodopsin, <i>N</i> -formyl peptide receptor, m3-muscarinic receptor	activated by β -subunits of G-proteins; expression of GRK3 is \approx 10% of GRK2	Benovic et al. (1991)	P26818
GRK4 (IT11)	57693	500 ^a	16 exons	4p16.3	testis, brain	palmitoylated	β 2-adrenergic receptor; chorioc gonadotropin receptor	—	Premont et al. (1996)	P32298
GRK5	67786	590	—	10q24	lung, heart, retina	unknown to be modified by a lipophilic group but regulated by lipids; autophosphorylated at Ser484 and Thr485; phosphorylated and inhibited by protein kinase C at the C-terminus	β 2-adrenergic receptor; thyrotropin receptor, photolyzed rhodopsin, m2-muscarinic receptor, neutral peptides, acidic proteins	associated with membranes	Kunapuli et al. (1994a); Premont et al. (1994)	P43249
GRK6	65969	576	—	5q35	brain, skeletal muscle, pancreas, heart, and lung	palmitoylated at the C-terminal region on one or more Cys residues (561, 562, and 565)	β 2-adrenergic receptor; m2-muscarinic receptor; acidic proteins; photolyzed rhodopsin; neutral peptides	lower activity than other GRKs toward standard <i>in vitro</i> substrates	Loudan & Benovic (1994); Stoffel et al. (1994)	P43250

^a Three additional splice variants.

This review will focus on the enzymological properties of GRKs.

GRKs are single-subunit kinases that phosphorylate activated GPCR

Six members of related mammalian Ser/Thr protein kinases have been cloned (Fig. 2, Table 1). These enzymes are a single-subunit (molecular mass, 63–80 kDa), water soluble, and show 30–85% amino acid sequence identity between protein pairs (57–96% overall similarity). Based on sequence similarity, this family is further subdivided into two groups comprising closely related GRK2 and GRK3 in the first group, and GRK1, GRK4, GRK5 and GRK6 in the second group. Unrelated GRK1, GRK2 and GRK4 gene structures suggest that they are functional analogs but not homologs (Penn and Benovic, 1994; Premont et al., 1996; Khani et al., 1996), and different chromosomal localizations indicate the absence of the evolutionary pressure for maintaining chromosomal clustering (Table 1). GRK2, GRK3 (known also as β -adrenergic receptor kinases 1 and 2), and GRK6 (for which Gagnon and Benovic, 1997, also identified a transcriptionally inactive pseudogene on human chromosome 13) are ubiquitously expressed, in contrast to the more specific localization of GRK1 (retinal photoreceptor cells and pinealocytes), GRK4 (testis, brain), and GRK5 (heart, lung and retina). The role of GRK1 in photoreceptors is likely restricted to photo-transduction, while its function in the pineal gland is unknown (Zhao et al., 1997). Nonetheless, patterns of expression likely imply functional specialization of the GRKs.

The amino-terminal domain (≈ 180 amino acids) (Fig. 2) is related ($\approx 65\%$ similarity) among all GRKs, although the sequence identity is $<30\%$. Conserved amino acids are found throughout this region, suggesting that the secondary structure may be similar among GRKs. The N-terminal region may be involved in the interaction with GPCRs* (Palczewski et al., 1993), whereas in GRK4, 5, and 6, the amino-terminal domain may also be involved in binding of phosphatidylinositol 4,5-bisphosphate (PtdIns4,5P₂) (Pitcher et al., 1996).

The ATP-specific, catalytic domain is found in the middle of the sequence and is highly similar among GRKs ($\approx 60\%$ identity for group I and $\approx 40\%$ identity between groups I and II). This region is similar to other protein kinases, however, protein kinase C isoenzymes are more closely related ($\approx 40\%$ identity), suggesting a close evolutionary connection and perhaps similar regulation among the GRKs and protein kinase C isoenzymes.

The carboxyl-terminal domain (80–200 amino acids; ≈ 40 –60% sequence identity) is the site of autophosphorylation (GRK1, GRK4, GRK5, and GRK6) on Ser and Thr residues within the sequence KD(N)V(I)DI(V)XXFST(A)VKGV. This sequence is similar to a part of the first β -strand and β -turn in acylphosphatase which contains key residues of the active site (Pastore et al., 1992). This similarity suggests that the autophosphorylation sequence may have evolved to bind phosphate groups covalently or noncovalently. Instead of these phosphorylatable residues, GRK2 and GRK3 have acidic residues -DEED- which may complete the same function. The C-terminus also is the site of farnesylation (GRK1) or palmitoylation (GRK4, GRK6) (reviewed by Premont et al., 1995). In contrast, GRK2 and GRK3 have a pleckstrin homology domain involved in the interaction with the $\beta\gamma$ subunits of G-proteins (Haga and Haga, 1992; Koch et al., 1993) and various species of phospholipids (Pitcher et al., 1995a; DebBurman et al., 1995, 1996; Onorato et al., 1995). Several splice variants of GRK4 were identified but their functional differences are unclear (Table 1).

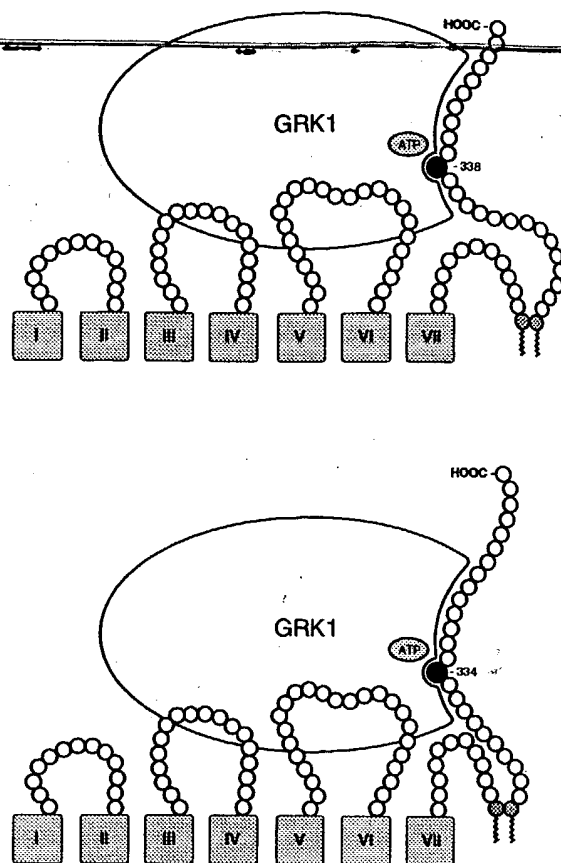


Fig. 3. The stable-complex model of phosphorylation of photolyzed rhodopsin by GRK1 at distinct sites. In this model, GPCR* forms stable complexes with GRK. Different conformations of the GPCR* C-terminus leads to phosphorylation at different residues of the receptor.

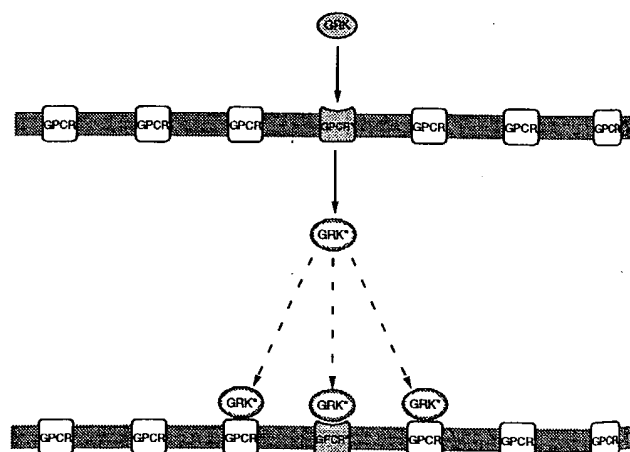


Fig. 4. The hysteresis model of the GRKs. In this model, GPCR* interacts with GRK producing an activated form of the kinase. GRK* is able to interact with GPCR and GPCR*.

GPCRs* are substrates for GRKs: two mechanistic models of GRK action

GPCRs exist in equilibrium between inactive and active conformations, which is shifted by the binding of agonist toward the active forms (GPCRs*). GRKs either phosphorylate only GPCRs* (according to a stable-complex model; Fig. 3), or they are activated by GPCRs* and then phosphorylate GPCRs* and

lesser degree GPCRs (according to a hysteresis model;

Fig. 4).

Several protein kinases phosphorylate peptides which contain an appropriate consensus sequence (a recognition motif). For example, protein kinase A shows preferences for the RRXS(T) sequence (Kemp and Pearson, 1991), suggesting that the substrate recognition site is contained within a continuous linear recognition element close to the phosphorylated residue. In contrast, GRKs recognize activated conformations of GPCRs rather than linear sequences. GRK1 and GRK2 (and most likely all other GRKs) phosphorylate peptides that encompass the phosphorylation region of the receptor, and also random acidic or neutral peptides containing Ser or Thr residues. The catalytic efficiency of peptide phosphorylation, however, is much lower, with a K_m as much as 10000 times higher and a V_{max} 10–100 times lower than for the activated receptor (Palczewski et al., 1989a; Onorato et al., 1991).

Akhtar and colleagues first demonstrated that GRK1 becomes activated as a result of its interaction with photolyzed rhodopsin and, in turn, can phosphorylate exogenously added peptide substrates (Fowles et al., 1988). The hysteresis model proposes that after interacting with GPCR*, GRK remains in the active conformation for a period of time (Brown et al., 1992). Activated kinase GRK* then is capable of dissociating from photolyzed rhodopsin and stays active with a half-life of ≈ 2 min (Dean and Akhtar, 1996), phosphorylating activated and non-activated receptors, or exogenous peptides (Fig. 4). The rate of phosphorylation of photolyzed rhodopsin by GRK1* is 80 times higher than the rate of phosphorylation of non-photolyzed rhodopsin (Dean and Akhtar, 1993). These observations are consistent with high-gain phosphorylation observed for rhodopsin, bleached by exceedingly low illumination (Aton, 1986; Binder et al., 1990).

The consequence of high-gain phosphorylation on phototransduction, if physiologically relevant, remains unclear. Due to the high concentration of rhodopsin in rod outer segments (≈ 3 mM), the phosphorylation of unbleached rhodopsin might be inconsequential for tempering the gain of G-protein activation by newly activated receptors (Binder et al., 1996). The photoreceptor response saturates at a very low fraction of bleached rhodopsin, and the ratio of phosphorylated rhodopsin/rhodopsin is exceedingly low; thus, the probability of the capture of light by phosphorylated rhodopsin is low. Alternatively, GRK1* may have a much higher affinity for unphosphorylated, photolyzed rhodopsin when compared to unactivated GRK1, thus effectively competing with G-protein activation and contributing to desensitization (light adaptation). The hysteresis phenomenon related to protein conformation is very rare in biochemistry. Typically, it involves a chemical reaction, covalent modification, and sudden changes in hydrostatic pressure or pH, making this enzymatic system intriguing for future studies.

It is possible that only GRK1 phosphorylates both active and unactivated GPCRs, displaying properties distinct from other GRKs. The argument would be that, due to low affinity for the site of phosphorylation, and the low density of GPCR (with the exception of rhodopsin), phosphorylation of non-activated GPCR by GRKs would occur with exceedingly low probability. Prolonged kinase activity would lead rather to the phosphorylation of other proteins (receptors) that are in high densities, owing to low sequence preference.

In contrast, the stable-complex model proposes that the kinase binds to GPCR*, and forms a stoichiometric and stable complex as determined by direct binding studies (Pulvermüller et al., 1993) or using dominant negative mutants of GRKs (Kong et al., 1994; Pals-Rylaarsdam et al., 1995). In the GRK1*·rhodopsin* complex, when extrapolated to physiological condi-

tions, GRK1 remains bound to rhodopsin* for >2 s (Hofmann et al., 1995). In this complex, the active site of the kinase is more efficient in catalysis, either using the C-terminus of its own receptor with which it forms a complex, or an exogenous peptide substrate (Palczewski et al., 1991; Chen et al., 1993) (Fig. 3). Truncated, photolyzed rhodopsin further enhances peptide phosphorylation as compared to photolyzed native rhodopsin, suggesting that the C-terminus may be an obstacle to the exogenous peptide. The half-saturating concentration for all truncated forms of rhodopsin in the stimulation of exogenous peptide are similar to the K_m for photolyzed rhodopsin, suggesting that the affinity of GRK1 for truncated, photolyzed rhodopsin is unchanged and involves mainly the cytoplasmic loops of the receptor (Fig. 3). Similarly, when comparable sites of the m2-muscarinic receptor are eliminated, the mutant can still activate GRK2 (Kameyama et al., 1994). However, the data derived from peptide studies should be considered with caution, since peptides may be phosphorylated through mechanisms other than GPCRs.

In this model, GRK1 first binds ATP, which increases the k_{on} for the activated receptor by a factor of ≈ 10 ($\approx 10 \mu M^{-1} s^{-1}$) (Pulvermüller et al., 1993). GRK recognizes the activated form of GPCR* generated as a result of the agonist binding. The binding of GRK to GPCR* may also take place due to activating mutations or protonation of acidic residues within transmembrane or cytoplasmic domains of GPCR, respectively (Pei et al., 1994; Rim and Oprian, 1995; Buczylo et al., 1996). The GPCR*·GRK complex involves geometrical complementaries of the N-terminal domain of the kinase and cytoplasmic loops of the receptor (for example Shi et al., 1995; Thurmond et al., 1997), allowing broad and overlapping substrate specificity (Table 1). Multiple Ser and Thr residues at the C-terminal region of many GPCRs are considered hallmarks for multiple phosphorylation, and *in vitro* multiple phosphorylation (5–9 phosphate groups/receptor molecule) has been observed for photolyzed rhodopsin, β_2 -adrenergic receptor, and other receptors. However, only limited, primary mono-phosphorylated or bis-phosphorylated species were detected *in vivo* (Benovic et al., 1986; Ohguro et al., 1995). These differences could result from the methods used for *in vitro* biochemical procedures, such as excess GRKs, lack of phosphatases, diluted membrane preparations with compromised membrane structure, strong receptor stimulation, long phosphorylation time courses, different milieus of nucleotides and metal ions, or diminished arrestin molecules and proteins involved in sequestration. Fig. 3 shows two complexes between photolyzed rhodopsin and GRK1, with an active site (with bound ATP) projecting toward residues 338 and 334. These two complexes differ by changes in the conformation of the C-terminus; however, it is possible that the sites of phosphorylation are influenced by a subtle change in the receptor's cytoplasmic loops, leading to a different projection of the active site toward the unchanged C-terminus. Note that either event assumes a weak interaction of the phosphorylated region with the catalytic site of GRK1, in contrast to the firm interaction of the kinase with the cytoplasmic loops. As reported for GRK1, GRK2 and GRK5, GRKs preferentially phosphorylate different Ser/Thr residues, using photolyzed rhodopsin or β_2 -adrenergic receptor as substrates (Palczewski et al., 1995; Fredericks et al., 1996). Multiple phosphorylation might be sequential, with a neighboring hydroxyl-containing amino acid adjoining the primary site of phosphorylation (Pullen et al., 1993; Pullen and Akhtar, 1994; Ohguro et al., 1993, 1996). This hierarchical phosphorylation scheme was also proposed for the phosphorylation of the N-formyl peptide receptor by GRK2 (Prossnitz et al., 1995). The k_{off} for the GPCR*·GRK complex is increased when either the kinase or receptor is phosphorylated (Pulvermüller et al., 1993).

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Both models predict that GRKs recognize activated conformations of the receptors, and that this interaction is less dependent on particular amino acid sequences. Several GRKs phosphorylate a large number of activated GPCRs (Table 1) with overlapping specificities but with different kinetics, thus resulting in different profiles of receptor desensitization. These properties make GRKs unique among the protein kinases, and exemplify in a broader sense, the importance of the inducible protein-protein interaction in controlling signaling activity, in addition to direct control of enzymatic activities by a diffusible ligand. Phosphorylation of GPCRs at different sites may play different roles in receptor physiology.

A different interpretation, involving protein kinase C, was proposed by Greene et al. (1997). This study is in contrast with the results obtained by Ohguro et al. (1996) and inconsistent with slow dark adaptation (resensitization) of human vision in patients lacking GRK1 (Yamamoto et al., 1997; Carr and Ripps, 1967).

GRKs might be regulated by calcium and lipids

GRKs are not directly affected by second messengers. However, this does not rule out the possibility of regulation by other proteins that respond to changes in the concentration of cAMP, Ca^{2+} , $\text{Ins}(1,4,5)\text{P}_3$, or phospholipids. Such regulation was proposed for GRK1 by the Ca^{2+} -binding protein, S-modulin (Kawamura, 1993), for GRK2 and GRK5 by protein kinase C (Chuang et al., 1995; Winstel et al., 1996; Pronin and Benovic, 1997), for GRK4 α and GRK5 by calmodulin (Chuang et al., 1996a; Sallese et al., 1997), for GRK2, 3, 4, 5 and 6 by phospholipids (for the most recent studies see DebBurman et al., 1996; Pitcher et al., 1996), and for the regulation of transcription of GRKs by second messengers (De Blasi et al., 1995; Iacovelli et al., 1996). The regulation of GRKs would produce branch points in signal transduction pathways. GRKs are also posttranslationally modified by prenylation and carboxymethylation (GRK1), palmitoylation (GRK4 and GRK6) and autophosphorylation (GRK1 and GRK5) (reviewed by Inglesse and Premont, 1996).

Farnesylation is not essential for bovine GRK1 to associate efficiently with its substrate, as the non-farnesylated mutant of GRK1 has significant activity toward photolyzed rhodopsin (Inglesse et al., 1992). Farnesylation is a permanent modification that may be part of a larger domain involved in the interaction with the membrane and is unlikely to function in dynamic regulation of the kinase. It is possible that farnesylation may play a vital role in directing GRK1 to a specific region of these highly differentiated rod and cone photoreceptors, thus retaining GRK1 tethered to membrane compartments of these cells. Consistent with this hypothesis is the observation that chicken rhodopsin kinase contains a consensus sequence for geranylgeranylation instead of farnesylation (Zhao, X. Y. and Palczewski, K., unpublished). Much less is known about the dynamics of the palmitoylation/palmitoylation regulation, and how this modification may influence the rate and the sites of GPCR* phosphorylation by GRK4 or GRK6, or their cellular localization (reviewed by Inglesse and Premont, 1996).

Autophosphorylation of GRK1 occurs predominantly on two neighboring residues, Ser487 and Thr488 (Palczewski et al., 1993), affecting the stabilization of the rhodopsin*-GRK1 complex (Pulvermüller et al., 1993) without changing the initial rate of phosphorylation. These results suggest that the competition between GRK1 and G-protein for the binding to GPCR* may be affected by autophosphorylation. In contrast, GRK5 undergoes phospholipid-stimulated autophosphorylation that increases its enzymatic activity by 15–20-fold. The binding of lipids was proposed to take place at the C-terminus of the enzyme, in the

vicinity of the autophosphorylation site (Kunapuli et al., 1994a). It is unclear if this lipid-binding site is in addition to the binding of $\text{PtdIns}(4,5)\text{P}_2$ at the N-terminus (Pitcher et al., 1996). GRK4 and GRK6 do not autophosphorylate in purified forms, even in the presence of phospholipids.

Several studies suggest that GRKs translocate from the cytosol to the membrane, for example, as a result of an agonist stimulation of GPCR, protein-kinase-C-dependent phosphorylation, binding of the G-protein $\beta\gamma$ -dimer, palmitoylation or farnesylation (Winstel et al., 1996; Inglesse et al., 1992). However, these experiments were done using biochemical and *in vitro* heterologous expression systems, therefore, the physiological significance of these observations remains unclear (Stoffel et al., 1997). In fact, GRK1 and GRK2, the most studied enzymes, were found to be localized to several membrane compartments of the cell (Palczewski et al., 1992; Garcia-Higuera et al., 1994; Murga et al., 1996). GRK4 and GRK5 are tightly associated with the membrane even in reconstitution assays and have increased basal activity toward receptors in the absence of an agonist. It is likely that GRKs are always membrane-associated and diffuse two-dimensionally to nearby receptors.

Regulation of GRK1 by recoverin involves direct complex formation between these two proteins (Chen et al., 1995a), likely blocking the kinase from interaction with photolyzed rhodopsin. Senin et al. (1997) found that recoverin inhibits the phosphorylation of unbleached rhodopsin more than it does that of bleached rhodopsin, proposing that this calcium regulation prevents GRK1 from participation in a side reaction (high-gain phosphorylation). Calmodulin strongly inhibits GRK4 α and GRK5. The enzymatic activity of GRK5 is unaffected when a soluble substrate was used, but calmodulin prevents association of GRK5 with membranes. GRK2 and 3 are much less affected by calmodulin (Chuang et al., 1996a; Haga et al., 1997). Intriguingly, three other splice variants of GRK4 (β , γ , and δ) are not inhibited by calmodulin (Sallese et al., 1997). These two calcium-binding proteins interact with their targets with different affinities, namely the EC_{50} between GRK1 and recoverin, GRK2, GRK4 α or GRK5 and calmodulin are $\approx 3 \mu\text{M}$, $\approx 10 \mu\text{M}$, $\approx 80 \text{ nM}$ and $\approx 40 \text{ nM}$, respectively. Thus, calcium through recoverin or calmodulin would prolong G-protein activation. It is interesting to note, however, that photoreceptor cells are unique as far as lowering calcium, while other cells show an increase in the calcium levels as a result of activation.

Additional controversy surrounding the physiological function of recoverin has been reviewed by Polans et al. (1996). Note that the phototransduction cascade produces a decreased influx of cations, including calcium. During the recovery, however, the drop in the intracellular free calcium remains even after the photo-current returns to the dark conditions (Gray-Keller and Detwiler, 1994), suggesting a fast buffering of entering calcium. The change in the initial phase of the photo-current is independent of rhodopsin phosphorylation (Chen et al., 1995b). Thus, the remaining time of the photoreponse occurs with low intracellular free calcium, during which phosphorylation takes place. It can be proposed that recoverin, in physiological conditions, may be a calcium buffer rather than being involved in GRK1 regulation (see Ohguro et al., 1996).

The activities of GRK2 and GRK5 are affected by some membrane phospholipids in which the native substrate, GPCRs, are inserted. For example, lipids bind to GRK2 and 5, inducing a more active conformation (Onorato et al., 1995; Kunapuli et al., 1994b). There are some conflicting data published on the lipid regulation of GRKs (DebBurman et al., 1996; Pitcher et al., 1995a, 1996; Kunapuli et al., 1994b), resulting likely from different experimental approaches. The addition of different lipids may stabilize the active conformation, GPCR*, to a varying

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(for example low level of Meta II formation in phosphatidylcholine vesicles). Since both the enzymes and their substrates are affected by lipids, a definitive answer to whether these described phenomena are direct effects on GRKs, GPCRs, or on the complex between both GRK-GPCR, awaits further experimentation.

Haga and collaborators found that $\beta\gamma$ subunits of G-proteins stimulate GRK2 (Haga and Haga, 1992; Haga et al., 1994a). The binding site was identified within the C-terminal pleckstrin homology domain (Pitcher et al., 1995a; Koch et al., 1993). Stimulation of GRK2 and 3 by the $\beta\gamma$ subunits might be dependent on the presence of phospholipids (DeBburman et al., 1996). G-protein-coupled receptors and G-protein $\beta\gamma$ subunits also show isoform-dependent specificity in GRK2 and GRK3 activation in intact cells (Daaka et al., 1997).

GRK2 is activated by protein-kinase-C-dependent phosphorylation (Chuang et al., 1995; Winstel et al., 1996). The site of phosphorylation has not been rigorously determined but is probably located within the pleckstrin homology domain. This activation might be analogous to the activation by $\beta\gamma$ subunits of G-proteins and phospholipids (increasing V_{max}/K_m ; Kim et al., 1993; Haga and Haga, 1992; Haga et al., 1994a). It remains to be determined how a combination of effector molecules (phospholipids, $\beta\gamma$ dimer of G-proteins, and protein kinase C) co-regulate this kinase in the cell. In contrast, protein kinase C stoichiometrically phosphorylates GRK5, resulting in an inhibition of GRK5. The site(s) of phosphorylation were localized to the C-terminal region of the kinase (Pronin and Benovic, 1997).

The information reviewed here can be summarized on three conclusions. First, GPCRs* exist in several quasi-stable conformations which can form distinct complexes with GRKs leading to phosphorylation at different sites. Second, the stable-complex model predicts that, in biochemical assays, mutation of one phosphorylatable Ser/Thr residue, for example to Ala, will cause phosphorylation of another residue in this same region, as Ser and Thr are typically present in a high frequency in proteins (already shown experimentally, Robinson et al., 1996). Third, phosphorylation at different sites may have important physiological consequences in serving different inactivation pathways of GPCR*. Due to significant progress in understanding the enzymology of GRKs, these predictions can be addressed in the future. Integration of these biochemical properties with events taking place under physiological conditions, including selectivity of kinases for a particular GPCR, however, remains a challenging area of research.

I thank Preston Van Hooser for help during the preparation of the manuscript. Drs Arthur Polans, Jim Inglese, Jack Saari, and Mike Gelb for valuable comments. This research was supported by a National Institute of Health grant (EY08161) and an award from Research to Prevent Blindness, Inc., to the Department of Ophthalmology at the University of Washington. The author is the recipient of a Jules and Doris Stein Research to Prevent Blindness (RPB) Professorship.

References

- Aton, B. R. (1986) Illumination of bovine photoreceptor membranes causes phosphorylation of both bleached and unbleached rhodopsin molecules, *Biochemistry* 25, 677–680.
- Bennett, N. & Sitaramayya, A. (1988) Inactivation of photoexcited rhodopsin in retinal rods: the roles of rhodopsin kinase and 48-kDa protein (arrestin), *Biochemistry* 27, 1710–1715.
- Benovic, J. L., Strasser, R. H., Caron, M. G. & Lefkowitz, R. J. (1986) β -Adrenergic receptor kinase: identification of a novel protein kinase that phosphorylates the agonist-occupied form of the receptor, *Proc. Natl Acad. Sci. USA* 83, 2797–2801.
- Benovic, J. L., Mayor, F. Jr, Staniszewski, C., Lefkowitz, R. J. & Caron, M. G. (1987) Purification and characterization of the β -adrenergic receptor kinase, *J. Biol. Chem.* 262, 9026–9032.
- Benovic, J. L., Onorato, J. J., Arriza, J. L., Stone, W. C., Lohse, M., Jenkins, N. A., Gilbert, D. J., Copeland, N. G., Caron, M. G. & Lefkowitz, R. J. (1991) Cloning, expression, and chromosomal localization of β -adrenergic receptor kinase 2. A new member of the receptor kinase family, *J. Biol. Chem.* 266, 14939–14946.
- Binder, B. M., Biernbaum, M. S. & Bownds, M. D. (1990) Light activation of one rhodopsin molecule causes the phosphorylation of hundreds of others. A reaction observed in electroporated frog rod outer segments exposed to dim illumination, *J. Biol. Chem.* 265, 15333–15340.
- Binder, B. M., O'Connor, T. M., Bownds, M. D. & Arshavsky, V. Y. (1996) Phosphorylation of non-bleached rhodopsin in intact retinas and living frogs, *J. Biol. Chem.* 271, 19826–19830.
- Böhm, S. K., Grady, E. F. & Bunnett, N. W. (1997) Regulatory mechanisms that modulate signalling by G-protein-coupled receptors, *Biochem. J.* 322, 1–18.
- Brown, N. G., Fowles, C., Sharma, R. & Akhtar, M. (1992) Mechanistic studies on rhodopsin kinase. Light-dependent phosphorylation of C-terminal peptides of rhodopsin, *Eur. J. Biochem.* 208, 659–667.
- Buczylo, J., Crouch, R., Saari, J. C. & Palczewski, K. (1996) Mechanisms of opsin activation, *J. Biol. Chem.* 271, 20621–20630.
- Carr, R. E. & Ripps, H. (1967) Rhodopsin kinetics and rod adaptation in Oguchi's disease, *Invest. Ophthalmol.* 6, 426–436.
- Chen, C.-Y., Dion, S. B., Kim, C. M. & Benovic, J. L. (1993) β -Adrenergic receptor kinase. Agonist-dependent receptor binding promotes kinase activation, *J. Biol. Chem.* 268, 7825–7831.
- Chen, C. K., Inglese, J., Lefkowitz, R. J. & Hurley, J. B. (1995a) Ca^{2+} -dependent interaction of recoverin with rhodopsin kinase, *J. Biol. Chem.* 270, 18060–18066.
- Chen, J., Makino, C. L., Peachey, N. S., Baylor, D. A. & Simon, M. I. (1995b) Mechanisms of rhodopsin inactivation *in vivo* as revealed by a COOH-terminal truncation mutant, *Science* 267, 374–377.
- Chuang, T. T., LeVine, H. 3rd & De Blasi, A. (1995) Phosphorylation and activation of β -adrenergic receptor kinase by protein kinase C, *J. Biol. Chem.* 270, 18660–18665.
- Chuang, T. T., Paolucci, L. & De Blasi, A. (1996a) Inhibition of G-protein-coupled receptor kinase subtypes by Ca^{2+} /calmodulin, *J. Biol. Chem.* 271, 28691–28696.
- Chuang, T. T., Iacovelli, L., Sallese, M. & DeBlasi, A. (1996b) G-protein-coupled receptors: Heterologous regulation of homologous desensitization and its implications, *Trends Pharmacol. Sci.* 17, 416–421.
- Daaka, Y., Pitcher, J. A., Richardson, M., Stoffel, R. H., Robishaw, J. D. & Lefkowitz, R. J. (1997) Receptor and G $\beta\gamma$ -isoform-specific interactions with G-protein-coupled receptor kinases, *Proc. Natl Acad. Sci. USA* 94, 2180–2185.
- Dean, K. R. & Akhtar, M. (1993) Phosphorylation of solubilized dark-adapted rhodopsin. Insights into the activation of rhodopsin kinase, *Eur. J. Biochem.* 213, 881–890.
- Dean, K. R. & Akhtar, M. (1996) Novel mechanism for the activation of rhodopsin kinase: implications for other G-protein-coupled receptor kinases (GRK's), *Biochemistry* 35, 6164–6170.
- DeBburman, S. K., Ptasienski, J., Boettcher, E., Lomasney, J. W., Benovic, J. L. & Hosey, M. M. (1995) Lipid-mediated regulation of G-protein-coupled receptor kinases 2 and 3, *J. Biol. Chem.* 270, 5742–5747.
- DeBburman, S. K., Ptasienski, J., Benovic, J. L. & Hosey, M. M. (1996) G-protein-coupled receptor kinase GRK2 is a phospholipid dependent enzyme that can be conditionally activated by G protein $\beta\delta$ -subunits, *J. Biol. Chem.* 271, 22552–22562.
- De Blasi, A., Parruti, G. & Sallese, M. (1995) Regulation of G-protein-coupled receptor kinase subtypes in activated T lymphocytes. Selective increase of β -adrenergic receptor kinase 1 and 2, *J. Clin. Invest.* 95, 203–210.
- Ferguson, S. S., Downey, W. E. 3rd, Colapietro, A. M., Barak, L. S., M'énard, L. & Caron, M. G. (1996a) Role of β -arrestin in mediating agonist-promoted G protein-coupled receptor internalization, *Science* 271, 363–366.
- Ferguson, S. S. G., Barak, L. S., Zhang, J. & Caron, M. G. (1996b) G-protein-coupled receptor regulation: role of G-protein-coupled recep-

- tor kinases and arrestins, *Can. J. Physiol. Pharmacol.* 74, 1095–1110.
- Fowles, C., Sharma, R. & Akhtar, M. (1988) Mechanistic studies on the phosphorylation of photoexcited rhodopsin, *FEBS Lett.* 238, 56–60.
- Fowles, C., Akhtar, M. & Cohen, P. (1989) Interplay of phosphorylation and dephosphorylation in vision: protein phosphatases of bovine rod outer segments, *Biochemistry* 28, 9385–9391.
- Fredericks, Z. L., Pitcher, J. A. & Lefkowitz, R. J. (1996) Identification of the G-protein-coupled receptor kinase phosphorylation sites in the human β_2 -adrenergic receptor, *J. Biol. Chem.* 271, 13796–13803.
- Gagnon, A. W. & Benovic, J. L. (1997) Identification and chromosomal localization of a processed pseudogene of human GRK6, *Gene* 184, 13–19.
- Garcia-Higuera, I., Penela, P., Murga, C., Egea, G., Bonay, P., Benovic, J. L. & Mayor, F. Jr (1994) Association of the regulatory β -adrenergic receptor kinase with rat liver microsomal membranes, *J. Biol. Chem.* 269, 1348–1355.
- Goodman, O. B. Jr, Krupnick, J. G., Santini, F., Gurevich, V. V., Penn, R. B., Gagnon, A. W., Keen, J. H. & Benovic, J. L. (1996) β -Arrestin acts as a clathrin adapter in endocytosis of the β_2 -adrenergic receptor, *Nature* 383, 447–450.
- Gray-Keller, M. P. & Detwiler, P. B. (1994) The calcium feedback signal in the phototransduction cascade of vertebrate rods, *Neuron* 13, 849–861.
- Greene, N. M., Williams, D. S. & Newton, A. C. (1997) Identification of protein kinase C phosphorylation sites on bovine rhodopsin, *J. Biol. Chem.* 272, 10341–10344.
- Haga, K. & Haga, T. (1992) Activation by G protein $\beta\gamma$ -subunits of agonist- or light-dependent phosphorylation of muscarinic acetylcholine receptors and rhodopsin, *J. Biol. Chem.* 267, 2222–2227.
- Haga, K., Kameyama, K. & Haga, T. (1994a) Synergistic activation of a G-protein-coupled receptor kinase by G protein $\beta\gamma$ -subunits and mastoparan or related peptides, *J. Biol. Chem.* 269, 12594–12599.
- Haga, T., Haga, K. & Kameyama, K. (1994b) G-protein-coupled receptor kinases, *J. Neurochem.* 63, 400–412.
- Haga, K., Tsuga, H. & Haga, T. (1997) Ca^{2+} -dependent inhibition of G-protein-coupled receptor kinase 2 by calmodulin, *Biochemistry* 36, 1315–1321.
- Hofmann, K. P., Jager, S. & Ernst, O. P. (1995) Structure and function of activated rhodopsin, *Israel J. Chem.* 35, 339–355.
- Iacovelli, L., Franchetti, R., Masini, M. & DeBlasi, A. (1996) GRK2 and β -arrestin1 as negative regulators of thyrotropin receptor-stimulated response, *Mol. Endocrinol.* 10, 1138–1146.
- Inglese, J., Koch, W. J., Caron, M. G. & Lefkowitz, R. J. (1992) Isoprenylation in regulation of signal transduction by G-protein-coupled receptor kinases, *Nature* 359, 147–150.
- Inglese, J., Freedman, N. J., Koch, W. J. & Lefkowitz, R. J. (1993) Structure and mechanism of the G-protein-coupled receptor kinases, *J. Biol. Chem.* 268, 23735–23738.
- Inglese, J. & Premont, R. T. (1996) Lipid modification of G-protein-coupled receptor kinases, *Biochem. Soc. Trans.* 24, 714–717.
- Kameyama, K., Haga, K., Moro, O. & Sadee, W. (1994) Activation of a GTP-binding protein and a GTP-binding-protein-coupled receptor kinase β -adrenergic-receptor kinase-1 by a muscarinic receptor m2 mutant lacking phosphorylation sites, *Eur. J. Biochem.* 226, 267–276.
- Kawamura, S. (1993) Rhodopsin phosphorylation as a mechanism of cyclic GMP phosphodiesterase regulation by S-modulin, *Nature* 362, 855–857.
- Kemp, B. E. & Pearson, R. B. (1991) Design and use of peptide substrates for protein kinases, *Methods Enzymol.* 200, 121–134.
- Khani, S. C., Abitbol, M., Yamamoto, S., Maravic-Magovcevic, I. & Dryja, T. P. (1996) Characterization and chromosomal localization of the gene for human rhodopsin kinase, *Genomics* 35, 571–576.
- Kim, C. M., Dion, S. B. & Benovic, J. L. (1993) β -Adrenergic receptor kinase. Agonist-dependent receptor binding promotes kinase activation, *J. Biol. Chem.* 268, 15412–15431.
- Koch, W. J., Inglese, J., Stone, W. C. & Lefkowitz, R. J. (1993) The binding site for the $\beta\gamma$ -subunits of heterotrimeric G proteins on the β -adrenergic receptor kinase, *J. Biol. Chem.* 268, 8256–8260.
- Kong, G., Penn, R. & Benovic, J. L. (1994) A β -adrenergic receptor kinase dominant negative mutant attenuates desensitization of the β_2 -adrenergic receptor, *J. Biol. Chem.* 269, 13084–13087.
- Krueger, K. M., Daaka, Y., Pitcher, J. A. & Lefkowitz, R. J. (1997) The role of sequestration in G-protein-coupled receptor resensitization. Regulation of β_2 -adrenergic receptor dephosphorylation by vesicular acidification, *J. Biol. Chem.* 272, 5–8.
- Kunapuli, P., Onorato, J. J., Hosey, M. M. & Benovic, J. L. (1994a) Expression, purification, and characterization of the G-protein-coupled receptor kinase GRK5, *J. Biol. Chem.* 269, 1099–1105.
- Kunapuli, P., Gurevich, V. V. & Benovic, J. L. (1994b) Phospholipid-stimulated autophosphorylation activates the G-protein-coupled receptor kinase GRK5, *J. Biol. Chem.* 269, 10209–10212.
- Lohse, M. J., Krasel, C., Winstel, R. & Mayor, F. Jr (1996) G-protein-coupled receptor kinases, *Kidney Int.* 49, 1047–1052.
- Loudon, R. P. & Benovic, J. L. (1994) Expression, purification, and characterization of the G-protein-coupled receptor kinase GRK6, *J. Biol. Chem.* 269, 22691–22697.
- Murga, C., Ruiz-Gómez, A., Garcia-Higuera, I., Kim, C. M., Benovic, J. L. & Mayor, F. Jr (1996) High affinity binding of β -adrenergic receptor kinase to microsomal membranes. Modulation of the activity of bound kinase by heterotrimeric G protein activation, *J. Biol. Chem.* 271, 985–994.
- Ohguro, H., Palczewski, K., Ericsson, L. H., Walsh, K. A. & Johnson, R. S. (1993) Sequential phosphorylation of rhodopsin at multiple sites, *Biochemistry* 32, 5718–5724.
- Ohguro, H., Van Hooser, J. P., Milam, A. H. & Palczewski, K. (1995) Rhodopsin phosphorylation and dephosphorylation *in vivo*, *J. Biol. Chem.* 270, 14259–14262.
- Ohguro, H., Rudnicka-Nawrot, M., Buczylo, J., Zhao, X., Taylor, J. A., Walsh, K. A. & Palczewski, K. (1996) Structural and enzymatic aspects of rhodopsin phosphorylation, *J. Biol. Chem.* 271, 5215–5224.
- Onorato, J. J., Palczewski, K., Regan, J. W., Caron, M. G., Lefkowitz, R. J. & Benovic, J. L. (1991) Role of acidic amino acids in peptide substrates of the β -adrenergic receptor kinase and rhodopsin kinase, *Biochemistry* 30, 5118–5125.
- Onorato, J. J., Gillis, M. E., Liu, Y., Benovic, J. L. & Ruoho, A. E. (1995) The β -adrenergic receptor kinase (GRK2) is regulated by phospholipids, *J. Biol. Chem.* 270, 21346–21353.
- Palczewski, K., Arendt, A., McDowell, J. H. & Hargrave, P. A. (1989a) Substrate recognition determinants for rhodopsin kinase: studies with synthetic peptides, polyanions, and polycations, *Biochemistry* 28, 8764–8770.
- Palczewski, K., Hargrave, P. A., McDowell, J. H. & Ingebritsen, T. S. (1989b) The catalytic subunit of phosphatase 2A dephosphorylates phosphopsin, *Biochemistry* 28, 415–419.
- Palczewski, K. & Benovic, J. L. (1991) G-protein-coupled receptor kinases, *Trends Biochem. Sci.* 16, 387–391.
- Palczewski, K., Buczylo, J., Kaplan, M. W., Polans, A. S. & Crabb, J. W. (1991) Mechanism of rhodopsin kinase activation, *J. Biol. Chem.* 266, 12949–12955.
- Palczewski, K., Buczylo, J., Van Hooser, P., Carr, S. A., Huddleston, M. J. & Crabb, J. W. (1992) Identification of the autophosphorylation sites in rhodopsin kinase, *J. Biol. Chem.* 267, 18991–18998.
- Palczewski, K., Buczylo, J., Lebiada, L., Crabb, J. W. & Polans, A. S. (1993) Identification of the N-terminal region in rhodopsin kinase involved in its interaction with rhodopsin, *J. Biol. Chem.* 268, 6004–6013.
- Palczewski, K. (1994) Structure and functions of arrestins, *Protein Sci.* 3, 1355–1361.
- Palczewski, K., Ohguro, H., Premont, R. T. & Inglese, J. (1995) Rhodopsin kinase autophosphorylation. Characterization of site-specific mutations, *J. Biol. Chem.* 270, 15294–15298.
- Pals-Rylaarsdam, R., Xu, Y., Witt-Enderby, P., Benovic, J. L. & Hosey, M. M. (1995) Desensitization and internalization of the m2 muscarinic acetylcholine receptor are directed by independent mechanisms, *J. Biol. Chem.* 270, 29004–29011.
- Pals-Rylaarsdam, R. & Hosey, M. M. (1997) Two homologous phosphorylation domains differentially contribute to desensitization and internalization of the m2 muscarinic acetylcholine receptor, *J. Biol. Chem.* 272, 14152–14158.
- Pastore, A., Saudek, V., Ramponi, G. & Williams, R. J. (1992) Three-dimensional structure of acylphosphatase. Refinement and structural analysis, *J. Mol. Biol.* 224, 427–440.
- Pei, G., Samama, P., Lohse, M., Wang, M., Codina, J. & Lefkowitz, R. J. (1994) A constitutively active mutant β_2 -adrenergic receptor is

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constitutively desensitized and phosphorylated, *Proc. Natl Acad. Sci. USA* 91, 2699–2702.

- Penn, R. B. & Benovic, J. L. (1994) Structure of the human gene encoding the β -adrenergic receptor kinase, *J. Biol. Chem.* 269, 14924–14930.
- Picher, J. A., Touhara, K., Payne, E. S. & Lefkowitz, R. J. (1995a) Pleckstrin homology domain-mediated membrane association and activation of the β -adrenergic receptor kinase requires coordinate interaction with G $\beta\gamma$ -subunit, *J. Biol. Chem.* 270, 11707–11710.
- Picher, J. A., Payne, E. S., Csontos, C., DePaoli-Roach, A. A. & Lefkowitz, R. J. (1995b) The G-protein-coupled receptor phosphatase: a protein phosphatase type 2A with a distinct subcellular distribution and substrate specificity, *Proc. Natl Acad. Sci. USA* 92, 8343–8347.
- Picher, J. A., Fredericks, Z. L., Stone, W. C., Premont, R. T., Stoffel, R. H., Koch, W. J. & Lefkowitz, R. J. (1996) Phosphatidylinositol 4,5-bisphosphate (PIP₂)-enhanced G-protein-coupled receptor kinase (GRK) activity, *J. Biol. Chem.* 271, 24907–24913.
- Polans, A. S., Baehr, W. & Palczewski, K. (1996) Turned on by Ca²⁺! The physiology and pathology of Ca²⁺-binding proteins in the retina, *Trends Neurosci.* 19, 547–554.
- Premont, R. T., Koch, W. J., Inglese, J. & Lefkowitz, R. J. (1994) Identification, purification, and characterization of GRK5, a member of the family of G-protein-coupled receptor kinases, *J. Biol. Chem.* 269, 6832–6841.
- Premont, R. T., Inglese, J. & Lefkowitz, R. J. (1995) G-protein-coupled receptor kinases, *FASEB J.* 9, 175–182.
- Premont, R. T., Macrae, A. D., Stoffel, R. H., Chung, N., Picher, J. A., Ambrose, C., Inglese, J., MacDonad, M. E. & Lefkowitz, R. J. (1996) Characterization of the G-protein-coupled receptor kinase GRK4. Identification of four splice variants, *J. Biol. Chem.* 271, 6403–6410.
- Pronin, A. N. & Benovic, J. L. (1997) Regulation of the G-protein-coupled receptor kinase GRK5 by protein kinase C, *J. Biol. Chem.* 272, 3806–3812.
- Prossnitz, E. R., Kim, C. M., Benovic, J. L. & Ye, R. D. (1995) Phosphorylation of the N-formyl peptide receptor carboxyl terminus by the G-protein-coupled receptor kinase, GRK2, *J. Biol. Chem.* 270, 1130–1137.
- Pullen, N., Brown, N. G., Sharma, R. P. & Akhtar, M. (1993) Cooperativity during multiple phosphorylations catalyzed by rhodopsin kinase: supporting evidence using synthetic phosphopeptides, *Biochemistry* 32, 3958–3964.
- Pullen, N. & Akhtar, M. (1994) Rhodopsin kinase: studies on the sequence of and the recognition motif for multiphosphorylations, *Biochemistry* 33, 14536–14542.
- Pulvermüller, A., Palczewski, K. & Hofmann, K. P. (1993) Interaction between photoactivated rhodopsin and its kinase: stability and kinetics of complex formation, *Biochemistry* 32, 14082–14088.
- Rim, J. & Oprian, D. D. (1995) Constitutive activation of opsin: interaction of mutants with rhodopsin kinase and arrestin, *Biochemistry* 34, 11938–11945.
- Robinson, P., Brannock, M. & Ke, W. (1996) The effects of rhodopsin phosphorylation cannot be duplicated with glutamic acid substitution at the phosphorylation sites, *Invest. Ophthalmol. Vis. Sci.* 37, 3723.
- Ruiz-Gomez, A. & Mayor, F. (1997) β -Adrenergic receptor kinase (GRK2) colocalizes with β -adrenergic receptors during agonist-induced receptor internalization, *J. Biol. Chem.* 272, 9601–9604.
- Sallese, M., Mariggio, S., Collodel, G., Moretti, E. & Piomboni, P., Baccetti, B. & DeBlasi, A. (1997) Protein-coupled receptor kinase GRK4 Molecular analysis of the four isoforms and ultrastructural localization in spermatozoa and germinal cells, *J. Biol. Chem.* 272, 10188–10195.
- Senin, I. I., Dean, K. R., Zargarov, A. A. & Akhtar, M. & Philippov, P. P. (1997) Recoverin inhibits the phosphorylation of dark-adapted rhodopsin more than it does that of bleached rhodopsin: A possible mechanism through which rhodopsin kinase is prevented from participation in a side reaction, *Biochem. J.* 321, 551–555.
- Shi, W., Osawa, S., Dickerson, C. D. & Weiss, E. R. (1995) Rhodopsin mutants discriminate sites important for the activation of rhodopsin kinase and Gt, *J. Biol. Chem.* 270, 2112–2119.
- Stoffel, R. H., Randall, R. R., Premont, R. T., Lefkowitz, R. J. & Inglese, J. (1994) Palmitoylation of G-protein-coupled receptor kinase, GRK6. Lipid modification diversity in the GRK family, *J. Biol. Chem.* 269, 27791–27791.
- Stoffel, R. H., Picher, J. A. & Lefkowitz, R. J. (1997) Targeting G protein-coupled receptor kinases to their receptor substrates, *J. Membr. Biol.* 157, 1–8.
- Thurmond, R. L., Creuzenet, C., Reeves, P. J. & Khorana, H. G. (1997) Structure and function in rhodopsin: Peptide sequences in the cytoplasmic loops of rhodopsin are intimately involved in interaction with rhodopsin kinase, *Proc. Natl Acad. Sci. USA* 94, 1715–1720.
- Tsuga, H., Kameyama, K., Haga, T., Kurose, H. & Nagao, T. (1994) Sequestration of muscarinic acetylcholine receptor m2 subtypes. Facilitation by G-protein-coupled receptor kinase (GRK2) and attenuation by a dominant-negative mutant of GRK2, *J. Biol. Chem.* 269, 32522–32527.
- Winstel, R., Freund, S., Krasel, C., Hoppe, E. & Lohse, M. J. (1996) Protein kinase cross-talk: membrane targeting of the β -adrenergic receptor kinase by protein kinase C, *Proc. Natl Acad. Sci. USA* 93, 2105–2109.
- Yamamoto, S., Sippel, K. C., Berson, E. L. & Dryja, T. P. (1997) Defects in the rhodopsin kinase gene in the Oguchi form of stationary night blindness, *Nat. Genet.* 15, 175–178.
- Zhang, J., Ferguson, S. S. G., Barak, L. S., Menard, L. & Caron, M. G. (1996) Dynamin and β -arrestin reveal distinct mechanisms for G-protein-coupled receptor internalization, *J. Biol. Chem.* 271, 18302–18305.
- Zhang, L. R., Sports, C. D., Osawa, S. & Weiss, E. R. (1997) Rhodopsin phosphorylation sites and their role in arrestin binding, *J. Biol. Chem.* 272, 14762–14768.
- Zhao, X. Y., Palczewski, K. & Ohguro, H. (1995) Mechanism of rhodopsin phosphorylation, *Biophys. Chem.* 56, 183–188.
- Zhao, X. Y., Haeseleer, F., Fariss, R. N., Huang, J. N., Baehr, W., Milam, A. H. & Palczewski, K. (1997) Molecular cloning and localization of rhodopsin kinase in the mammalian pineal, *Vis. Neurosci.* 14, 225–232.

Note added in proof. The stable-complex model is supported by the results of studies on rhodopsin (Zhang et al., 1997) and the m2 muscarinic acetylcholine receptor (Pals-Rylaarsdam and Hosey, 1997).

THE ROLE OF RECEPTOR KINASES AND ARRESTINS IN G PROTEIN-COUPLED RECEPTOR REGULATION

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KEY WORDS: phosphorylation, agonist-specific desensitization, sequestration, rhodopsin,
 β_2 -adrenergic receptor

ABSTRACT

G protein-coupled receptors (GPRs) play a key role in controlling hormonal regulation of numerous second-messenger pathways. However, following agonist activation, most GPRs rapidly lose their ability to respond to hormone. For many GPRs, this process, commonly referred to as desensitization, appears to be primarily mediated by two protein families: G protein-coupled receptor kinases (GRKs) and arrestins. GRKs specifically bind to the agonist-occupied receptor, thereby promoting receptor phosphorylation, which in turn leads to arrestin binding. Arrestin binding precludes receptor/G protein interaction leading to functional desensitization. Many GPRs are then removed from the plasma membrane via clathrin-mediated endocytosis. Recent studies have implicated endocytosis in the resensitization of GPRs and have linked both GRKs and arrestins to this process. In this review, we discuss the role of GRKs and arrestins in regulating agonist-specific signaling and trafficking of GPRs.

AGONIST-SPECIFIC DESENSITIZATION OF G PROTEIN-COUPLED RECEPTORS

In order to respond to rapid changes in agonist concentration, activated receptors must be inactivated and then restored for stimulation. In intracellular

signaling systems, the effects of an agonist are regulated at several levels, but alterations at the receptor locus appear to be the major means by which regulation occurs in many G protein-coupled receptor (GPR) signaling pathways. Acute regulation at the level of the GPR, called desensitization, is characterized by the waning of a stimulated response in the presence of continuous agonist exposure, and this phenomenon has been demonstrated in many different hormone and neurotransmitter signaling systems and in many different organisms. The phenomenon of desensitization has also been referred to as quenching, deactivation, tolerance, adaptation, and tachyphylaxis.

The phenomenon of desensitization can be subdivided along several lines: agonist-specific or non-agonist-specific, depending on the nature of the causative stimulus; rapid (seconds to minutes) or slow (hours to days); and loss of receptor signaling function (uncoupling) or loss of receptor number (down regulation). Agonist-specific desensitization is characterized by the loss of GPR responsiveness to the desensitizing agent only, while non-agonist-specific desensitization is characterized by diminished responsiveness to multiple stimuli following exposure to a specific desensitizing agent. A hallmark of agonist-specific desensitization is the normally rapid, functional uncoupling of GPR/G protein interaction at a time when the total pool of receptors remains essentially constant. The functional uncoupling of GPR/G protein interaction is perceived as a reduction in the sensitivity to and maximal effect of agonist. Desensitization of the response to light by the photoreceptor rhodopsin and the response to hormone by the β_2 -adrenergic receptor (β_2 AR) are the best-studied model systems for this phenomenon (1, 2).

Since rhodopsin is available in greater quantities than any other GPR, much of our knowledge on the phenomenon of desensitization has come from studies with rhodopsin. In order to perceive continuous environmental changes in light, the rod cell photoreceptor GPRs must recover from light activation and be restored for subsequent stimulation. Thus, a rapid turnoff mechanism has evolved for terminating phototransduction. In phototransduction, desensitization of rhodopsin was observed to occur in less than 1 s following light stimulation (3), thereby preventing a brief flash of light from being perceived as continuous illumination.

The β_2 AR was one of the first nonvisual GPRs to be purified in substantial quantities, and it also demonstrated very marked desensitization. Within a few seconds of β -agonist exposure, detectable increases in cAMP occurred, but this increase plateaued or even returned to near basal levels within a few minutes (4–6). Desensitization of the β_2 AR was exemplified by the observation that plasma membrane preparations derived from desensitized cells exhibit markedly diminished adenylyl cyclase activity upon restimulation with agonist. The rapid loss of β_2 AR-stimulated adenylyl cyclase activity occurred

without a change in the ability to activate the G protein directly, indicating that the adaptation was not occurring at the level of the G protein. Desensitization was postulated to occur via alteration of the β_2 AR itself, since purified β AR from desensitized turkey erythrocytes showed an impaired capacity to stimulate adenylyl cyclase when assessed in a reconstituted system (7).

While rhodopsin and the β_2 AR have served as useful model systems, agonist-specific desensitization is a general phenomenon that has been observed for many GPRs. In this review, we discuss the role of G protein-coupled receptor kinases (GRKs) and arrestins in regulating agonist-specific signaling of GPRs.

G PROTEIN-COUPLED RECEPTOR KINASES

The light-dependent phosphorylation of rhodopsin *in vivo* was discovered in the early 1970s (8, 9). It was subsequently demonstrated that the kinetics of rhodopsin phosphorylation correlate well with the quenching of cGMP phosphodiesterase (PDE) activity (10), indicating that rhodopsin phosphorylation likely is involved in its desensitization. Using ion exchange chromatography to separate highly phosphorylated rhodopsin into species with different extents of phosphorylation, Wilden & Kuhn (11) showed that $\geq 70\%$ of the rhodopsin molecules had ≥ 7 mol/mol phosphorylation stoichiometry, while the remainder had between 0 and 7 mol/mol, suggesting cooperativity between individual phosphorylation sites.

The GRK that phosphorylates rhodopsin, called rhodopsin kinase or GRK1, binds preferentially to light-activated rhodopsin (12), a characteristic that was exploited in its purification (13). Upon binding to light-activated rhodopsin, rhodopsin kinase phosphorylates the receptor at multiple Ser and Thr residues (8, 9). Rhodopsin kinase also phosphorylates the cone-specific pigment iodopsin, suggesting that a rhodopsin kinase-like GRK regulates cone cell photoreceptors (14). Rhodopsin kinase was subsequently cloned (15) and was demonstrated to be predominantly expressed in retina, where it localized to both rods and cones, and to a lesser extent in the pineal body (16). Rhodopsin kinase contains a consensus CAAX motif to direct its farnesylation (17, 18), a modification that likely targets rhodopsin kinase to the membrane where rhodopsin is located.

Peptide mapping analysis of phosphorhodopsin (19) and lack of phosphorylation of a C-terminally truncated rhodopsin (20) indicated that most of the phosphorylation sites in rhodopsin are located in its C terminus. Interestingly, the C terminus of rhodopsin appears to become more accessible in the metarhodopsin II state (21), the activated state of rhodopsin that is phosphorylated by rhodopsin kinase, suggesting a conformational change in rhodopsin upon its light-activation. Many subsequent studies *in vitro* identified Ser338

and Ser343 as the primary phosphorylation sites in the C terminus (22–24). In vivo, Ser334, Ser338, and Ser343 were demonstrated to be the major sites of phosphorylation of rhodopsin (25,26). The observations that rhodopsin and C-terminally truncated rhodopsin stimulate rhodopsin kinase phosphorylation of exogenous peptide substrates suggested a model in which inactive rhodopsin kinase binds to metarhodopsin II and becomes activated to phosphorylate C-terminal residues (20,27).

Phosphorylation of rhodopsin greatly reduces PDE activation, and the magnitude of this effect correlated with the stoichiometry of phosphorylation (28), prompting speculation that phosphorylation of rhodopsin directly reduces its binding affinity for transducin. The role of rhodopsin phosphorylation in inactivating rhodopsin in vivo was elegantly demonstrated using transgenic mice expressing a C-terminal truncation mutant of rhodopsin (terminating at Ser334). Abnormally prolonged responses were recorded from rods of these transgenic mice, attributed to activation of the truncated rhodopsin species that lack most of the C-terminal phosphorylation sites (29).

A covalent modification of the β AR upon its desensitization was suggested by a decrease in the electrophoretic mobility of the desensitized β AR (30). Phosphorylation was identified as the covalent modification, since stoichiometric phosphorylation of the β_2 AR occurred after exposure of intact cells to agonist and the kinetics and dose response of β_2 AR phosphorylation paralleled those of desensitization (31,32). A kinase other than the cAMP-dependent protein kinase (PKA) (involved in non-agonist-specific desensitization) was implicated in agonist-specific desensitization of the β AR because agonist-induced phosphorylation and desensitization still occurred in kin^- S49 lymphoma cells, which lack PKA, and cyc^- cells, which lack the α subunit of G_s (4,32,33).

A partially purified preparation of a protein kinase from the supernatant of a high-speed spin of lysed kin^- S49 lymphoma cells was capable of phosphorylating the agonist-occupied β_2 AR, indicating that this protein kinase may indeed be involved in agonist-specific desensitization of the β_2 AR (34). A cDNA encoding this protein kinase (termed β -adrenergic receptor kinase, β ARK, or GRK2) was subsequently cloned and sequenced, and it expressed an enzyme that preferentially phosphorylates the agonist-activated β_2 AR (35). Unlike the restricted localization of rhodopsin kinase, β ARK is expressed ubiquitously (35,36). Moreover, β ARK purified from bovine brain was demonstrated to have a K_d of 0.25 μM for the agonist-activated β_2 AR, phosphorylating it to a stoichiometry of ~ 8 mol/mol (36). Interestingly, β ARK can phosphorylate light-activated rhodopsin and rhodopsin kinase can phosphorylate the agonist-activated β_2 AR, suggesting that the mechanisms regulating these two signaling systems are similar (37). Binding to the cytosolic domains of GPRs also appears to activate β ARK, which is similar to the proposed mechanism of activation

of rhodopsin kinase (38). Initial studies suggested that the β ARK phosphorylation sites on the β_2 AR are also located in its Ser/Thr-rich C terminus (39). More recent studies have identified serines 396, 401, and 407 and threonine 384 as the primary sites for β ARK phosphorylation of the human β_2 AR (40). In fact, nearly all GPRs have clusters of Ser/Thr residues in the third intracellular domain and/or C terminus that are potential phosphorylation sites for GRKs.

The significant amino acid sequence homology between rhodopsin kinase and β ARK (~33% identity and ~58% similarity) suggested that they were members of a gene family of GRKs (15, 35). In the early 1990s four other members of the GRK family were discovered. β ARK2 (GRK3) was cloned from a bovine brain cDNA library (41), IT11 (GRK4) from a human frontal cortex cDNA library (42), GRK5 from a human heart cDNA library (43), and GRK6 from human heart and brain cDNA libraries (44). Two *Drosophila melanogaster* GRKs, GPRK1 and GPRK2, were also cloned in the early 1990s (45). While β ARK, β ARK2, GRK5, and GRK6 are ubiquitous, GRK4 is found predominantly in testes (Table 1). All GRKs share a similar structural organization with a poorly conserved N-terminal domain of ~185 residues, a conserved protein kinase catalytic domain of ~270 residues, and a variable length C-terminal domain of 105–230 residues (Figure 1).

Biochemical characterization of the GRKs was greatly enhanced by baculovirus-mediated overexpression in Sf9 insect cells. Sf9-expressed β ARK and β ARK2 specifically phosphorylate the agonist-occupied form of rhodopsin ($K_m \sim 10 \mu\text{M}$) and the β_2 AR ($K_m \sim 50 \text{ nM}$) (46). Many studies subsequently demonstrated that two important regulators of β ARK and β ARK2 are the $\beta\gamma$ subunits of G proteins (47–49) and acidic phospholipids (50–53). The specific binding site for $\beta\gamma$ subunits on β ARK was localized to a discrete region in its C terminus (largely within the pleckstrin homology domain) (54). The

Table 1 Molecular properties of G protein-coupled receptor kinases

Family name	Common name	Membrane association	Tissue distribution	Chromosome mapping	Features/regulation
GRK1	RK ^a	Farnesylation	Retina	13q34	Autophosphorylation
GRK2	β ARK1	$G_{\beta\gamma}$, Acidic PL	Ubiquitous	11q13	PH domain, PKC, calmodulin
GRK3	β ARK2	$G_{\beta\gamma}$, Acidic PL	Ubiquitous	22q11	PH domain
GRK4	IT-11	Palmitoylation	Testis	4q16.3	Four splice variants
GRK5		PL Binding	Ubiquitous	10q24-qter	Autophosphorylation, PKC, calmodulin
GRK6		Palmitoylation	Ubiquitous	5q35	Calmodulin

^aRK, rhodopsin kinase; PL, phospholipid; PH, pleckstrin homology.

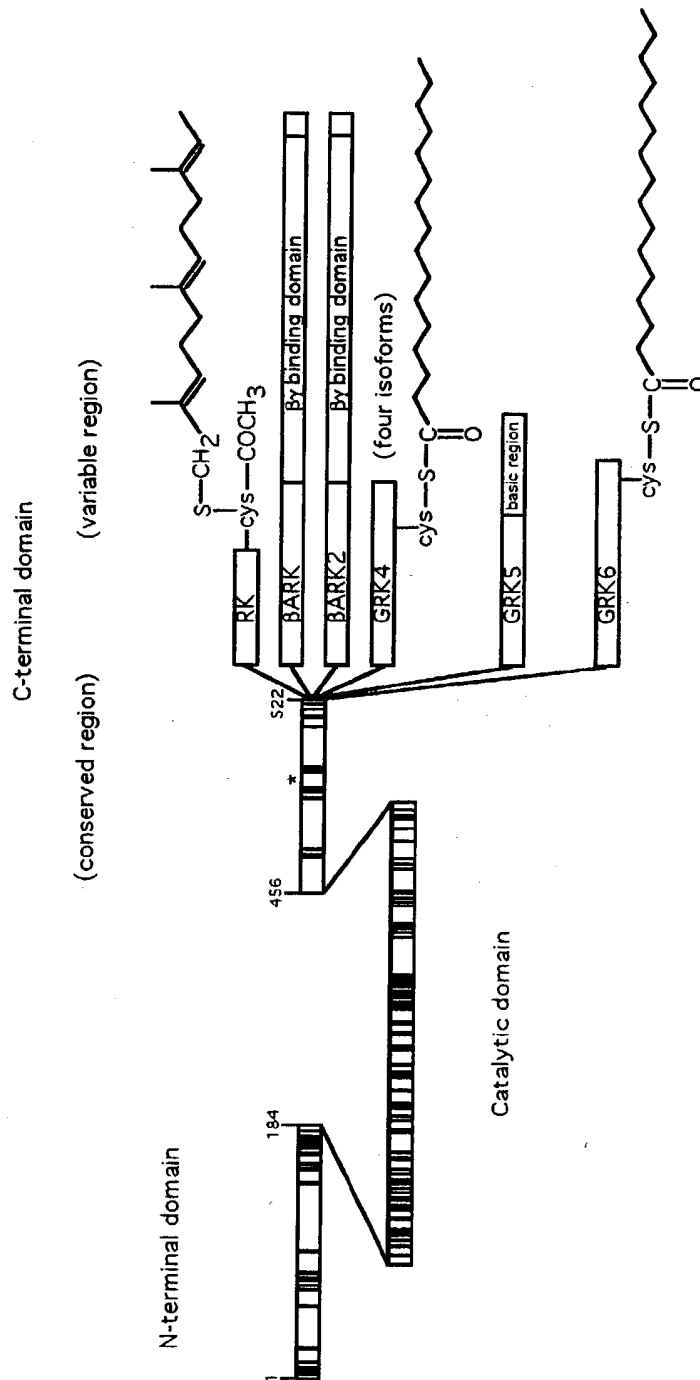


Figure 1 Domain architecture of G protein-coupled receptor kinases (GRKs). The sequences of the six known mammalian GRKs are represented schematically. The solid areas are regions of invariant amino acid sequence. The bifurcation in the C-terminal domain represents divergence in sequence between the various GRKs, where rhodopsin kinase (RK) is farnesylated, β -adrenergic receptor kinase (BARK) and $\beta\gamma$ binding domain, GRK4 and GRK6 are palmitoylated, and GRK5 contains a basic phospholipid binding domain.

other GRKs utilize additional mechanisms to promote membrane association, an event critical for receptor interaction. Rhodopsin kinase is farnesylated (17, 18), GRK4 (55) and GRK6 (56) are palmitoylated, and GRK5 binds to phospholipids via polybasic regions in the N- and C-terminal domains (57, 58) (Figure 1). Recent studies have demonstrated that palmitoylation of GRK6 plays an important role in membrane association of the kinase, thereby enhancing its ability to phosphorylate receptor substrates (59).

Another characteristic that appears specific for the GRK subtype involves regulation of kinase activity. For example, rhodopsin kinase has been shown to be inhibited by the Ca^{2+} -binding protein recoverin (60). Calcium binding to recoverin promotes its association with the kinase, inactivating it and thereby reducing its ability to phosphorylate rhodopsin. Since calcium levels are decreased upon light activation of rod cells (61), recoverin/rhodopsin kinase interaction might provide a mechanism for adaptation of the system to ambient light. Because recoverin has no effect on β ARK, regulation by recoverin may be specific for rhodopsin kinase (60). Recent studies also have demonstrated that β ARK and GRK5 are subject to regulatory phosphorylation via protein kinase C (PKC), a Ca^{2+} /phospholipid-dependent kinase. β ARK phosphorylation by PKC leads to an approximately two- to threefold activation of the kinase, possibly via an increased ability of β ARK to bind to membranes (62, 63). In contrast, GRK5 is inhibited severalfold when phosphorylated by PKC because of both a decreased activity and a decreased affinity for receptor (64). Several recent studies have also implicated calmodulin in the calcium-dependent regulation of GRKs (65–67). Calmodulin is a potent inhibitor of GRK activity with a specificity for GRK5 ($\text{IC}_{50} \sim 50 \text{ nM}$) > GRK6 \gg β ARK ($\text{IC}_{50} \sim 2 \mu\text{M}$) \gg rhodopsin kinase. The calmodulin-binding domain of GRK5 has been localized within the N-terminal domain of the kinase (67), a region also implicated in phospholipid binding (58).

In addition to the β_2 AR, several other GPRs were shown or suggested to be phosphorylated by GRKs, including the m1 muscarinic cholinergic (mACh) (68), m2 mACh (69), m3 mACh (70), α_2 -adrenergic (71), angiotensin $_{1A}$ (72), substance P (73), prostaglandin E_1 (74), somatostatin (75), and olfactory (76) receptors. Although the GRKs phosphorylate multiple receptors in vitro, some studies demonstrated specificity among GPR substrates (77–80).

Several different lines of evidence have directly implicated GRK phosphorylation in desensitization of GPRs. Coexpression of GRKs with GPRs in heterologous cells resulted in enhanced desensitization of the β_2 -adrenergic (81), β_1 -adrenergic (82), α_{1B} -adrenergic (83), α_2 -adrenergic (79), thrombin (84), angiotensin II_{1A} (72), A3 adenosine (85), m2 mACh (86), and luteinizing hormone/chorionic gonadotropin (55) receptors. Deletion or mutation of putative phosphorylation sites in the GPR cytoplasmic domains impaired desensitization

of the α_{2A} -adrenergic (87), thrombin (84), m2 mACh (88), β_2 -adrenergic (89), luteinizing hormone/chorionic gonadotropin (90), neurokinin 2 (91), α_{1B} -adrenergic (92), and platelet-activating factor (93) receptors. Inhibition of β ARK activity with heparin diminished desensitization of the α_{2A} -adrenergic (87), β_2 -adrenergic (94), and olfactory (78) receptors. Finally, coexpression of a dominant-negative β ARK (containing a mutation that inhibits its catalytic activity) inhibited desensitization of the m2 mACh (88), angiotensin II_{1A} (72), β_2 -adrenergic (95), β_1 -adrenergic (82), α_{1B} -adrenergic (83), δ -opioid (96), and A2 adenosine (97) receptors. Agonist-dependent phosphorylation also accompanied desensitization of the cAMP receptor in *Dictyostelium discoideum* (98) and the α -mating factor receptor in yeast (99).

In vivo evidence that GRKs are involved in desensitizing GPRs comes from both characterization of transgenic mice overexpressing specific GRKs and antisense strategies. Transgenic mice with cardiac overexpression of β ARK were demonstrated to have blunted inotropic and chronotropic responses to isoproterenol, as well as reduced sensitivity to and maximal effect of β AR-stimulated adenylyl cyclase activity in myocardial membranes (100). Similarly, cardiac-specific overexpression of GRK5 resulted in marked β AR desensitization upon agonist stimulation (101). Interestingly, in the GRK5 transgenic mice, the response to angiotensin II stimulation was unchanged, while in the β ARK transgenic mice, the response to angiotensin II was attenuated (101), thus demonstrating specificity of GRKs in vivo. Finally, utilizing antisense GRK constructs, a role for β ARK in agonist-induced desensitization of the β_2 -adrenergic (102) and H₂ histamine (103) receptors and GRK5 in agonist-induced desensitization of the thyrotropin receptor (104) have been demonstrated in intact cells.

ARRESTINS

The arrestins are a class of soluble proteins that function in concert with the GRKs to stop or arrest intracellular signaling. In the continued presence of stimulus, arrestins ensure that each activated GPR is turned off first before reinitiating signal transduction. The prototypic arrestin is a 48-kDa protein, originally called S-antigen, that was first discovered as a causative antigen of experimental autoimmune uveitis, a degenerative eye disease. Retinal S-antigen was subsequently demonstrated to be the 48-kDa protein regulating light-dependent signal transduction in rod photoreceptor cells (thus its name visual arrestin, also referred to as arrestin) (105).

Visual arrestin was initially characterized as a major protein that redistributed from the cytosol to the rod outer segment (ROS) disk membrane following light-activation of rhodopsin (106), and affinity purification of arrestin was described based on its light-dependent binding to rhodopsin (107). A bovine

arrestin cDNA was subsequently cloned and demonstrated to encode a protein of 404 amino acids (108, 109). Arrestin is highly restricted in localization, being found most abundantly in retinal tissue and the developmentally related pineal gland (110). Another retinal-specific arrestin protein was more recently cloned and found to be ~50% homologous to arrestin (111, 112). This arrestin also demonstrated a highly restricted expression pattern, being enriched in cone photoreceptors in the retina (thus its name cone arrestin) (111, 112). Cone arrestin is also referred to as X-arrestin because its gene is localized on the X-chromosome. Based on the restricted localization of arrestin and cone arrestin predominantly to the retina, it is inferred that these proteins specifically regulate photoreceptor GPRs. The conserved function of arrestins is highlighted by the cloning of two *D. melanogaster* visual arrestin genes (113, 114).

The light-dependent binding of arrestin to rhodopsin is highly enhanced by rhodopsin phosphorylation. Arrestin is soluble in the dark but associates with ROS disk membranes when rhodopsin absorbs light and is phosphorylated by rhodopsin kinase (106). It was further shown that arrestin binding to rhodopsin absolutely requires rhodopsin to be both phosphorylated and in its active metarhodopsin II state (3). The affinity of arrestin/rhodopsin interaction was estimated to be ~50 nM based on the ability of arrestin to stabilize metarhodopsin II (3). Characterization of the arrestin/rhodopsin interaction was greatly facilitated by the *in vitro* translation of arrestin using a rabbit reticulocyte lysate translation system (115). *In vitro* translated arrestin bound specifically to the phosphorylated light-activated form of rhodopsin, exhibiting ~10–12-fold better binding to phosphorylated light-activated rhodopsin than either light-activated or phosphorylated dark rhodopsin (115). Importantly, *in vitro* translated arrestin binding to rhodopsin exhibited a similar affinity as purified bovine arrestin binding to rhodopsin (115). Moreover, *in vitro* translated arrestin bound preferentially to rhodopsin compared to the β_2 -adrenergic and m2 mACh receptors (116, 117).

Many studies have demonstrated that while phosphorylation of rhodopsin alone modestly desensitizes rhodopsin signaling, arrestin is required to fully quench signaling and accelerate the recovery process (118, 119). The mechanism of arrestin quenching of rhodopsin signaling was postulated to be via a binding competition between arrestin and transducin for phosphorylated light-activated rhodopsin, based on the observation that a crude ROS extract containing transducin displaces arrestin from phosphorylated ROS membranes (106). In support of this hypothesis, it was shown that arrestin binding to rhodopsin stabilizes the active metarhodopsin II state but prevents rhodopsin from coupling to transducin (3). Moreover, recent direct binding studies using purified preparations of arrestin and transducin have demonstrated that these proteins do indeed directly compete for binding to phosphorylated light-activated rhodopsin (120). The function and specificity of arrestin was shown in *in vitro* reconstitution

experiments where an ~1:1 molar ratio of arrestin:phosphorylated light-activated rhodopsin inhibits 50% of rhodopsin/transducin interaction, while ~100-fold higher concentrations were required for the same effect with phosphorylated β_2 AR (110, 121). Interestingly, a truncated form of arrestin (lacking the last 35 amino acids and called p44) was identified and shown to strongly inhibit transducin activation by nonphosphorylated light-activated rhodopsin (122). Furthermore, localization of p44 to ROSs indicates a possible role for this form of arrestin in regulating phototransduction (123).

A role for arrestin in regulation of rhodopsin function *in vivo* was demonstrated in studies that genetically manipulated arrestin levels in *D. melanogaster* photoreceptors. Interestingly, in the absence of arrestins, photoreceptors manifested light-dependent retinal degeneration likely due to the continuously activated state of the photoreceptor GPRs (124). Moreover, in a more recent study, Xu et al (125) demonstrated that in the absence of arrestins, desensitization occurred at ~50% of the level of desensitization obtained in the presence of arrestin. Interestingly, this study is consistent with the observation that phosphorylation of rhodopsin alone reduced transducin binding and activation by ~30–50% (120). Arrestins also appear to be associated with degenerative retinitis pigmentosa caused by constitutively active rhodopsin mutants. In this case, phosphorylation of constitutively active rhodopsin mutants by rhodopsin kinase and subsequent arrestin binding apparently led to sequestration of the entire pool of arrestin, resulting in the appearance of a constitutively activated state of photoreceptor GPRs upon subsequent exposure to light (126).

The potential role of an auxiliary protein in regulation of hormonal transduction mediated by the β AR was suggested by the ability of a partially purified β ARK preparation to inhibit up to ~80% of β_2 AR signaling, while more highly purified β ARK preparations only modestly inhibited signaling (127). These observations indicated that a cofactor in the partially purified preparation enhanced the inactivating effects of β ARK. This protein was postulated to be an arrestin-like protein, as visual arrestin potentiated the inactivating effects of β ARK on β_2 AR signaling (127). Lohse et al (110) subsequently cloned the cDNA for β -arrestin and found that it encoded a protein of 418 amino acids that is highly homologous to arrestin. Another nonvisual arrestin, called β -arrestin2 or arrestin3, was cloned from bovine brain (128), human thyroid (129), and rat brain (130) and found to encode a protein of 409 amino acids. β -arrestin and arrestin3 exist as polypeptide variants with short and long forms of each having been identified (~8–11 amino acid differences) (128). Overall, the arrestins are 45% identical and 70% similar between residues 16 and 349 of visual arrestin (Figure 2). Similar to most of the GRKs, β -arrestin and arrestin3 are ubiquitous (110, 128, 130). While β -arrestin appears to be the major nonvisual arrestin expressed in many tissues (128), arrestin3 is the predominant form in

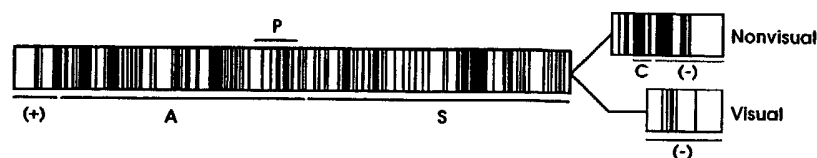


Figure 2 Domain architecture of arrestins. The sequences of the four known mammalian arrestins are represented schematically. The *solid areas* are regions of invariant amino acid sequence. The bifurcation near the C terminus represents divergence in sequence between the visual (arrestin and cone arrestin) and nonvisual (β -arrestin and arrestin3) arrestins. "A" represents the activation-recognition domain, "P" the phosphorylation-recognition domain, "S" the secondary hydrophobic interaction domain, "C" the clathrin binding domain, "+" the basic amino terminus, and "-" the acidic C terminus.

olfactory epithelium (77). The ubiquitous expression patterns of β -arrestin and arrestin3 suggest that unlike visual arrestin and cone arrestin, these arrestins have a relatively broad receptor specificity (Table 2).

Similar to visual arrestin, *in vitro* translation of β -arrestin and arrestin3 have greatly enhanced characterization of their GPR binding properties. *In vitro* translated β -arrestin bound to the m2 mAChR in a phosphorylation-dependent manner, with highest binding occurring to the agonist-activated phosphorylated form of the receptor (116). *In vitro* translated β -arrestin and arrestin3 modestly discriminated between the m2 mACh and β_2 -adrenergic receptors with K_d values of 0.3–0.6 nM (117). Moreover, purified β -arrestin from Sf9 insect cells bound to the β_2 AR in a phosphorylation- and agonist-dependent manner, exhibiting a K_d of ~ 2 nM and a stoichiometry of one β -arrestin per receptor (131). In contrast to visual arrestin, β -arrestin and arrestin3 interact quite well with phosphorylated non-agonist-activated GPRs (117, 131).

The functional effect of nonvisual arrestins on GPR signaling has been demonstrated in many studies. A partially purified preparation of β -arrestin

Table 2 Molecular properties of arrestins

Name	Receptor specificity ^a	Tissue distribution	Chromosome mapping	Features/regulation
Arrestin	$\rho \gg \beta_2\text{AR} \sim \text{m2 mAChR}$	Rod cells	2q37	Three splice variants
β -Arrestin	$\beta_2\text{AR} \sim \text{m2 mAChR} > \rho$	Ubiquitous	11q13	Two splice variants, clathrin binding
Arrestin3	$\beta_2\text{AR} \sim \text{m2 mAChR} > \rho$	Ubiquitous	17p13	Two splice variants, clathrin binding
Cone arrestin	Unknown	Cone cells	Xcen-q21	Unknown

^a ρ , rhodopsin.

inhibited the ability of β ARK-phosphorylated β_2 AR, but not phosphorylated rhodopsin, to activate G_s by >75% (110), indicating that β -arrestin works in concert with β ARK to effect agonist-specific desensitization of the β_2 AR. It was further shown in reconstitution experiments with purified proteins that a 1–1.5:1 molar ratio of β -arrestin or arrestin3:phosphorylated β_2 AR results in 50% inhibition of G_s activation, and ~20–100-fold higher amounts of β -arrestin or arrestin3 were required for inhibition of rhodopsin-stimulated transducin activation (121, 130). These results confirm the specificity of GPR interaction observed in in vitro binding studies. Importantly, the ability of β -arrestin to inhibit β_2 AR/ G_s coupling was enhanced dramatically by β ARK phosphorylation of the β_2 AR but not PKA phosphorylation of the β_2 AR (121, 132), a modification which also uncouples the β_2 AR from G_s (132, 133). The concerted action of β -arrestin and β ARK on inhibiting β_2 AR-stimulated adenylyl cyclase activity was also demonstrated using cell membranes from A431 cells (131).

A role for arrestins in homologous desensitization of GPR signaling in vivo has been illustrated by several studies. Addition of arrestin3-specific polyclonal antibodies to permeabilized olfactory cilia blocked desensitization of odorant receptors (77). Moreover, in intact cells, coexpression of β -arrestin or arrestin3 enhanced desensitization of the β_2 -adrenergic (81), β_1 -adrenergic (82), and α_{1B} -adrenergic (83) receptors. Finally, coexpression of β -arrestin synergistically enhanced the functional desensitization of the m2 mAChR by β ARK (86). Interestingly, in the latter study overexpression of β -arrestin alone with the m2 mAChR did not enhance receptor desensitization in JEG-3 cells, suggesting specifically that GRKs are limiting in JEG-3 cells and more generally that the role of GRKs and arrestins in regulation of GPR signaling in different cells is likely dependent on both the receptor and cell type.

Molecular Nature of GPR/Arrestin Interactions

The observation that arrestins discriminate between the agonist-activated and non-agonist-activated state of GPRs suggests that these molecules contain a domain(s) that specifically contacts those regions of GPRs that manifest their activated state (likely to be in the cytoplasmic domains of the GPR). Initial localization of this region (called the activation-recognition region) was provided by the observation that a truncated in vitro translated arrestin containing only the N-terminal half of the molecule (residues 1–191) partially retained the ability to recognize the light-activated state of rhodopsin (115). Moreover, the far C terminus was not likely to be involved in this function because p44 arrestin interacts very well with light-activated rhodopsin (122). It was further demonstrated that at least three regions within the N-terminal half of arrestin are involved in recognizing the light-activated state of rhodopsin (134). The N-terminal half of β -arrestin and arrestin3 also retained the ability to recognize

the agonist-activated state of GPRs, indicating that the activation-recognition region of all arrestins is likely contained within the N-terminal half (116, 117) (Figure 2). Utilizing synthetic peptides from the cytoplasmic sequence of bovine rhodopsin, Krupnick et al (135) demonstrated that visual arrestin likely interacts with the third, and to a lesser extent with the first, cytoplasmic loop rhodopsin sequences and that these regions of rhodopsin are likely the counterpart for the activation-recognition region(s) of arrestin. Moreover, direct binding studies using various GST-receptor fusion constructs demonstrated that the third cytoplasmic loops of the m2 and m3 mAChR and α_2 AR specifically bind the nonvisual arrestins (136).

The ability of arrestins to discriminate between the phosphorylated and non-phosphorylated states of the GPR indicates that there is also a region(s) of arrestins that specifically contacts the corresponding portion of the GPR that is phosphorylated upon agonist activation. It was initially postulated that this region (called the phosphorylation-recognition region) is a cationic region, since heparin and other polyanions compete efficiently with phosphorylated light-activated rhodopsin for arrestin binding (137). Direct evidence that the phosphorylation-recognition region of arrestin is located in its N-terminal half was provided by the observation that a truncated form of arrestin containing only its N-terminal half (residues 1–191) retains the ability to recognize the phosphorylated state of rhodopsin (115). Moreover, the N-terminal half of β -arrestin and arrestin3 also retained the ability to recognize the phosphorylated state of the m2 mAChR (116, 117).

Extensive mutagenesis of arrestin subsequently localized the phosphorylation-recognition region to a small discrete domain in the N terminus (Figure 2). While arrestin truncated at residue 185 bound to phosphorylated light-activated rhodopsin and phosphorylated dark rhodopsin, arrestin truncated at residue 158 demonstrated a significantly reduced capacity to recognize the phosphorylated form of rhodopsin, thus localizing the site between residues 158 and 185 (134). Consistent with this localization was the observation that an antibody to residues 170–182 of arrestin did not recognize it when bound to phosphorylated light-activated rhodopsin, and a 170–182 peptide from arrestin competed for arrestin binding to phosphorylated light-activated rhodopsin (138). Furthermore, proteolysis of the C terminus of phosphorylated rhodopsin reduced interaction with the 170–182 arrestin peptide, strongly indicating that this region of arrestin interacts with the phosphorylated C terminus of rhodopsin (138). Mutagenesis of individual residues within region 158–185 of arrestin identified several basic residues critical for phosphate interaction, mainly Arg171, Arg175, and Lys176 (139). Moreover, Arg175 appears to function as a phosphorylation-sensitive trigger, since neutralization or reversal of this charge resulted in a constitutively active (phosphorylation-independent) arrestin that has high affinity for nonphosphorylated light-activated rhodopsin (139).

A conformational change in arrestin upon its binding to phosphorylated light-activated rhodopsin (regulating its strict binding selectivity) was initially suggested by the observation that binding of arrestin to phosphorylated light-activated rhodopsin proceeds with a large Arrhenius activation energy (3). A conformational change in arrestin was also indicated by an increase in its sensitivity to limited proteolysis when bound to phosphorylated light-activated rhodopsin (137). Identification of the activation-recognition and phosphorylation-recognition regions of arrestin (primary interaction sites) led to a model for this conformational change in which simultaneous engagement of these regions in arrestin with the corresponding regions in phosphorylated light-activated rhodopsin results in a conformational change that exposes a secondary hydrophobic binding site (located between residues 191 and 365) for high-affinity interaction (134) (Figure 2). Indeed, the involvement of hydrophobic interactions is supported by the ability of salt to stimulate arrestin binding to phosphorylated light-activated rhodopsin (134). Consistent with this model, a synthetic phosphopeptide from the C terminus of rhodopsin promoted arrestin binding to light-activated nonphosphorylated forms of rhodopsin, indicating that a major function of rhodopsin phosphorylation is to stimulate arrestin binding to other regions on the cytoplasmic surface of light-activated rhodopsin (140).

A role for the C terminus of arrestin in regulating the putative conformational change was suggested by the ability of C-terminally truncated arrestin and p44 to bind well to both phosphorylated dark rhodopsin and light-activated rhodopsin, thus demonstrating little selectivity for phosphorylated light-activated rhodopsin (115, 122, 141). Further mutagenesis suggested that an intramolecular interaction between the basic N terminus and acidic C terminus of arrestin regulates its selectivity for binding to phosphorylated light-activated rhodopsin (116, 117, 142). Thus, the C terminus likely maintains a rigid structure of arrestin (possibly by interacting with its own N terminus) until rhodopsin is both agonist-activated and phosphorylated, upon which arrestin/rhodopsin interactions may replace intramolecular arrestin interactions (141). The lack of selectivity of C-terminally truncated arrestins for phosphorylated light-activated rhodopsin, therefore, is likely due to the failure of these mutant arrestins to maintain a rigid structure, leading to aberrant accessibility of the secondary hydrophobic interaction site.

Since arrestin and β -arrestin display different selectivity for binding to GPRs, arrestin/ β -arrestin chimeras were instrumental in uncovering regions of arrestins critical for directing GPR binding specificity. At least one domain involved in conferring GPR binding specificity is located in the N-terminal half of β -arrestin (116). More extensive analysis of arrestin/ β -arrestin chimeras subsequently indicated that two large centrally located domains in arrestin (between residues 48 and 365) and β -arrestin (between residues 45 and 367) confer

specificity of binding to GPRs (117). Importantly, exchanging the N- and C-terminal ends of arrestin and β -arrestin did not dramatically alter GPR binding specificity, consistent with the likely role of these regions in regulating the conformation of arrestins (117).

AGONIST-INDUCED INTERNALIZATION OF GPRs

Another level of regulation of GPRs is their internalization or sequestration following agonist exposure. Numerous studies have demonstrated that agonist exposure promotes the translocation of GPRs from the cell surface to an intracellular compartment distinct from the cell surface. Sequestration was thus originally defined as the agonist-induced internalization of GPRs from the plasma membrane to an ill-defined cytosolic compartment such that the GPRs are physically sequestered from the membrane-bound G protein.

The rapid redistribution of cell surface β_2 ARs was initially discovered when it was observed that agonist treatment of bullfrog erythrocytes results in a decrease in cell surface β_2 ARs with a concomitant increase in intracellular β_2 ARs (143). Moreover, early ligand-binding studies with the β_2 AR demonstrated a progressive loss of high-affinity binding of agonists during the course of the binding assay, suggesting a loss of β_2 ARs from the plasma membrane (144, 145). The internalized β_2 ARs were not accessible to hydrophilic ligands but were accessible to hydrophobic ligands (146), thus providing a means for measuring β_2 AR sequestration. Sequestered β_2 ARs were found to be associated with a "light vesicle" fraction that could be separated from a "heavy vesicle" fraction (plasma membrane) by sucrose density gradient fractionation (147). The light vesicle fraction was deficient in plasma membrane markers and contained distinct hydrodynamic and permeability properties, indicating physical separation of the β_2 AR from the plasma membrane (147–149).

Sequestered β_2 ARs displayed a reduced binding affinity for agonists and a lack of effect of guanine nucleotides on agonist binding (147), both hallmarks for absence of GPR/G protein coupling. The translocation of β_2 ARs from the plasma membrane to an intracellular compartment occurred very rapidly, exhibiting a $t_{1/2}$ of ~ 2 min after a delay of ~ 1 min (149). β_2 AR sequestration was apparently independent of β_2 AR/ G_s coupling (150), and the only requirement for sequestration appeared to be agonist occupancy of the β_2 AR, since a direct correlation between β_2 AR occupancy and sequestration was observed (94). Perhaps the best study to date on the sequestration of the β_2 AR used immunocytochemistry to investigate the subcellular receptor localization. Using conventional and confocal fluorescence microscopy, it was demonstrated that the rapid agonist-induced translocation of the β_2 AR into the cytosol of HEK293 cells (within 2 min of agonist exposure, the β_2 AR redistributed in

small intracellular punctate accumulations) temporally parallels sequestration of the β_2 AR measured by radioligand binding (151).

Several lines of evidence have suggested that the agonist-induced translocation of the β_2 AR from the plasma membrane to intracellular vesicles occurs via the clathrin-coated pit/endosome pathway employed by constitutively recycling receptors (i.e. low-density lipoprotein and transferrin receptors) and growth factor receptors [i.e. epidermal growth factor (EGF) receptor]. Incubation of astrocytoma cells with EGF and isoproterenol resulted in comigration of the EGF receptor and β_2 AR to low-density regions of sucrose gradients, suggesting that these receptors are processed in parallel (152). Moreover, techniques that disrupt internalization through clathrin-coated pits (i.e. hypertonicity treatment, cytosolic acidification, intracellular potassium depletion, reduced temperature, and reduced cellular ATP) also disrupted internalization of the β_2 AR (153–155). The rapid kinetics of β_2 AR internalization also suggested an endocytic pathway involving clathrin-coated pits (156). Finally, immunocytochemical subcellular localization of the β_2 AR following agonist treatment demonstrated colocalization with the transferrin receptor, suggesting that sequestered β_2 ARs undergo processing in a manner similar to that for the constitutively recycling transferrin receptor (151).

Radioligand binding and immunohistochemical techniques have also demonstrated an agonist-induced translocation of other GPRs from the plasma membrane to an intracellular site. These GPRs include the various mACh (157), μ - and δ -opioid (158), and 5-hydroxytryptamine_{2A} (159) receptors. Similar techniques as described above for the β_2 AR also were used to demonstrate that the mACh (160, 161), δ - and μ -opioid (158, 162), 5-hydroxytryptamine_{2A} (159), and α_{1B} -adrenergic (163) receptors internalize via clathrin-coated pits. Moreover, a temperature-sensitive clathrin heavy chain mutant was used to demonstrate that the yeast pheromone receptor internalizes via clathrin-coated pits (164). To date, there is no evidence that the photoreceptor rhodopsin undergoes light-induced internalization, consistent with the localization of rhodopsin to the ROS disk membrane, a site that is already internalized relative to the plasma membrane.

Internalization of GPRs as a Possible Mechanism for Desensitization

Sequestration of the β_2 AR was originally proposed to be a mechanism for desensitization based on the observation that the light membrane fraction containing sequestered β_2 ARs did not contain G_s (149). Moreover, intraperitoneal injection of isoproterenol into rats resulted in rapid desensitization of the β AR accompanied by translocation of ~40% of the receptors from the plasma membrane to a light vesicle fraction (165). Similar studies also demonstrated that rapid β_2 AR desensitization was associated with a loss of cell surface receptors

(166). Finally, by applying specific inhibitors to digitonin-permeabilized A431 cells, Lohse et al (94) demonstrated that sequestration of the β_2 AR alone contributes ~20–30% to desensitization of this GPR.

While there are studies indicating that sequestration of the β_2 AR may be linked to its rapid agonist-induced desensitization, it is clear that sequestration alone cannot fully explain desensitization. In fact, many studies suggested that sequestration of the β_2 AR does not even contribute to its desensitization. For example, inhibiting β_2 AR sequestration through clathrin-coated pits did not inhibit its rapid agonist-induced desensitization (152–155, 167). Moreover, several studies demonstrated that the rapid agonist-induced desensitization of the β_2 AR occurs more rapidly than its internalization (148, 149, 168). Lastly, several studies demonstrated that the extent of sequestration of the β_2 AR is not as great as the extent of reduction in agonist-stimulated adenylyl cyclase activity (5, 94). Taken together, these studies thus distinguish the rapid initial uncoupling of β_2 AR/ G_s interaction from β_2 AR sequestration.

Several studies have suggested that sequestration of other GPRs contributes to their desensitization. For example, mutation of C-terminal Thr residues in the m3 mAChR severely impaired its sequestration and its ability to desensitize (160). Moreover, alteration of receptor density at the cell surface appeared to be the major determinant for the observed agonist-induced desensitization of the μ -opioid receptor (169). The majority of studies, however, indicate that these two phenomena are distinct because the processes of desensitization and internalization of the angiotensin_{1A} (170), D1 dopamine (171), m2 mACh (88), secretin (172), neurokinin 1 (173), and histamine H2 (174) receptors were independent.

Internalization of GPRs as a Possible Mechanism for Resensitization

Although GPR sequestration may not be essential for rapid agonist-induced desensitization, recent studies have indicated that it may play a role in accelerating recovery from desensitization (a process commonly referred to as resensitization). Several studies indirectly demonstrated that internalization is involved in resensitization of the β_2 AR. While treatment with isoproterenol resulted in rapid sequestration of the β_2 AR, internalized receptors were found to return to the cell surface following removal of agonist (146, 149, 175). Moreover, during agonist-induced steady state redistribution, the β_2 AR appears to undergo repetitive endocytosis and recycling back to the plasma membrane (176). Finally, sequestered β_2 ARs were structurally intact and capable of stimulating adenylyl cyclase when subsequently reconstituted (177, 178).

It was directly shown that sequestration of the β_2 AR promotes its resensitization, since inhibition of β_2 AR internalization through clathrin-coated pits with either concanavalin A or sucrose prevented resensitization (167, 179). It

was subsequently proposed that β_2 AR sequestration restores its function by enabling a vesicle-derived phosphatase to dephosphorylate the β_2 AR, following which the dephosphorylated functional β_2 AR is recycled back to the cell surface (167). Interestingly, early studies actually suggested this model when it was observed that sequestered β_2 ARs were phosphorylated to a lesser degree (~ 0.75 mol/mol stoichiometry) than the total cellular pool of β_2 AR (~ 2.1 mol/mol stoichiometry), and the light vesicle fraction was shown to contain a potent phosphatase activity capable of dephosphorylating β_2 ARs previously phosphorylated by β ARK (180). The critical importance to β_2 AR resensitization of both a phosphatase activity and subsequent recycling of functional receptors back to the plasma membrane was demonstrated by the ability of calyculin A, an inhibitor of protein phosphatases, and monensin, an inhibitor of intracellular trafficking, to block resensitization of the β_2 AR (167).

Recently, a protein phosphatase capable of dephosphorylating β ARK-phosphorylated β_2 AR (called G protein-coupled receptor phosphatase or GRP) was purified from bovine brain and shown to be a latent oligomeric form of protein phosphatase 2A (181). It was further postulated that a specific conformation of the β_2 AR, induced by intracellular vesicular acidification, was required for GRP to dephosphorylate the β_2 AR, since disruption of endosomal acidic pH blocks association of the β_2 AR with GRP and subsequent β_2 AR dephosphorylation (182). A general model for the role of agonist-induced sequestration in regulation of β_2 AR function is schematically illustrated in Figure 3.

Other studies have also suggested a role for internalization of GPRs in resensitization. Agonist exposure of α_{1B} -ARs resulted in rapid internalization, and the internalized GPRs were reactivated and continuously recycled back to the plasma membrane during agonist exposure (163). Moreover, endocytosis of the neurokinin 1 receptor was demonstrated to be critical for its resensitization (173). Similar to the β_2 AR, both a phosphatase activity and recycling of functional receptors back to the plasma membrane were found to be critical for resensitization of the neurokinin 1 receptor (173). Internalization of the thrombin receptor also appears to be central to its resensitization but in a slightly unique manner involving an already internalized, golgi-localized intracellular pool of thrombin receptors. Agonist stimulation of the thrombin receptor induced its internalization and subsequent targeting to lysosomes while the intracellular pool of thrombin receptors was translocated to the cell surface to replenish functional receptors (183).

Unlike many GPRs, internalization of the photoreceptor rhodopsin likely does not occur and is not a mechanism for its resensitization. A few studies have shed light on the unique manner in which rhodopsin is resensitized. Arrestin was found to inhibit dephosphorylation of freshly photoactivated rhodopsin by protein phosphatase 2A, suggesting that arrestin dissociation must occur before dephosphorylation of rhodopsin and may in fact regulate this process

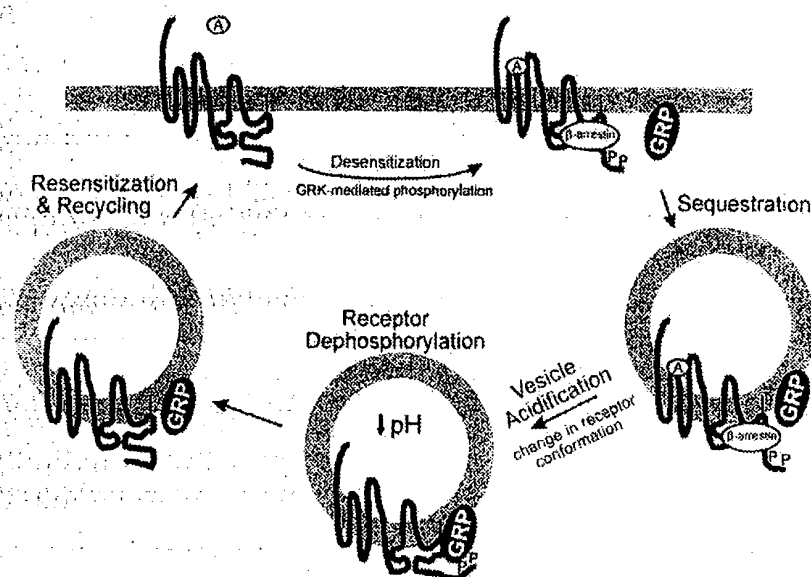


Figure 3 Model depicting the desensitization, sequestration, and resensitization of the β -adrenergic receptor (β_2 AR). Upon agonist binding, the receptor is initially desensitized by G protein-coupled receptor kinase (GRK)-mediated phosphorylation and β -arrestin binding. The β -arrestin-bound receptor is then targeted to clathrin-coated pits where it is endocytosed. Endosomal acidification promotes receptor and G protein-coupled receptor phosphatase (GRP) association and dephosphorylation of the receptor. The receptor then recycles back to the cell surface where it can again be activated by agonist. "A" represents agonist and "PP" the sites of GRK phosphorylation on the receptor. (Reprinted with permission from 182.)

(184). In order to recycle rhodopsin for phototransduction, not only must arrestin dissociate from rhodopsin, but the bleached retinal chromophore must also be replaced. It was demonstrated that reduction of all-*trans*-retinal to all-*trans*-retinol by retinol dehydrogenase followed by dissociation of all-*trans*-retinol from rhodopsin and rebinding of 11-*cis*-retinal were essential for recycling of rhodopsin (185). Thus, the current model for resensitization of rhodopsin at the ROS disk membrane involves reduction of all-*trans*-retinal (metarhodopsin II state) to all-*trans*-retinol, dissociation of all-*trans*-retinol from rhodopsin, dissociation of arrestin from opsin (removal of arrestin blockade), dephosphorylation of opsin, regeneration of the chromophore (rebinding of 11-*cis*-retinal), and dissociation of the rhodopsin phosphatase.

Role of GRKs and Arrestins in Internalization of GPRs

Early studies on the β AR were inconclusive on the role of phosphorylation in sequestration of this GPR. Truncation of the C terminus of the β AR at residue 365 or 354 (removing some or all putative β ARK phosphorylation sites,

respectively) did not affect its sequestration (89, 186). In contrast, truncation proximal to residue 354 (also removing all putative β ARK phosphorylation sites) significantly impaired sequestration of the β_2 AR (187). Moreover, a β_2 AR mutant containing mutation of four putative β ARK phosphorylation sites in the C terminus was defective not only in agonist-induced phosphorylation and desensitization but also in sequestration (188).

Evidence is now accumulating that agonist-induced phosphorylation is likely important for the internalization of many GPRs. A Ser/Thr-rich sequence was postulated to play an important role in sequestration of the m1, m2, and m3 mAChRs (189). Moreover, either truncation or mutation of the Ser/Thr residues in the C terminus of the thrombin receptor inhibited both its agonist-induced phosphorylation and sequestration (190). In addition, C-terminal deletions or point mutations of Ser/Thr residues in the C terminus of the δ -opioid receptor significantly reduced its agonist-induced internalization (162).

Recent studies have directly demonstrated a role for GRKs in agonist-induced sequestration of GPRs. β ARK phosphorylation of the m2 mAChR enhanced its sequestration, and coexpression of the m2 mAChR with a dominant-negative β ARK decreased sequestration (191). The direct role of phosphorylation of the β_2 AR in its sequestration was demonstrated using a phosphorylation- and sequestration-defective β_2 AR (β_2 AR-Y326A) (192). Overexpression of β ARK with β_2 AR-Y326A enhanced its phosphorylation and sequestration (193). Moreover, in addition to β ARK, GRKs 3–6 enhanced phosphorylation and sequestration of β_2 AR-Y326A with the agonist-dependent rescue of phosphorylation correlating with the rescue of internalization (194). Interestingly, coexpression with GRK4 and GRK6 resulted in agonist-independent phosphorylation of β_2 AR-Y326A accompanied by an increase in basal receptor sequestration (194). Further evidence that β ARK directly plays a role in β_2 AR sequestration was provided by the observation that dominant-negative β ARK attenuates both phosphorylation and sequestration of coexpressed wild-type β_2 AR (193).

It is now becoming evident that the ability of GRKs to promote sequestration of GPRs is dependent on the arrestins. Overexpression of β -arrestin or arrestin3 alone with β_2 AR-Y326A promoted its sequestration, and this effect was enhanced by β ARK coexpression (195). Moreover, mutants of β -arrestin (V53D) and arrestin3 (V54D) inhibited the ability of β ARK to rescue sequestration of β_2 AR-Y326A and inhibited sequestration of coexpressed wild-type β_2 AR (195). These mutant arrestins have thus been referred to as dominant-negative proteins. The role of both GRKs and arrestins in regulating sequestration of GPRs was highlighted by recent observations that β_2 AR sequestration in different cell types apparently correlates best with the expression levels of both β ARK and β -arrestin (196) and that sequestration of the m2 mAChR is synergistically regulated by both β ARK and β -arrestin (86). Interestingly,

coexpression of the angiotensin_{1A} receptor, a GPR that does not normally internalize via clathrin-coated pits, with β -arrestin promoted its internalization through the classic clathrin-mediated endocytic pathway (197). These results thus clearly establish a direct role for arrestins in internalization of GPRs and suggest that this effect occurs through clathrin-coated pits.

Recent studies have demonstrated that arrestins can both desensitize agonist-activated GPRs and promote their sequestration by interacting not only with the GPR but also with clathrin, the major protein component of the clathrin-based endocytic machinery (198). It was observed that β -arrestin and arrestin3, but not visual arrestin, interact specifically, stoichiometrically, and with high affinity and rapid kinetics with clathrin. Moreover, immunofluorescence analyses demonstrated that the activated β_2 AR, β -arrestin, and clathrin all colocalize in intact cells upon agonist addition, suggesting that the arrestin/clathrin interaction observed *in vitro* also occurs in cells in the presence of an activated receptor. Thus, β -arrestin and arrestin3 appear to act as a signal for internalization of agonist-activated GPRs by virtue of their ability to target the desensitized receptor to clathrin-coated pits. By interacting with high affinity with both receptors and clathrin, the nonvisual arrestins thus fulfill the requisite functional properties of an adaptor protein. In light of the many studies demonstrating that internalization is intricately involved in regulating GPR activity as well as many studies demonstrating that arrestins interact with multiple GPRs, it is tempting to speculate that many other GPR signaling systems will utilize a similar mechanism of arrestin-promoted internalization of agonist-activated phosphorylated receptors through clathrin-coated pits.

The predominant clathrin binding domain in nonvisual arrestins has been localized to hydrophobic and acidic residues between amino acids 367 and 385 in arrestin3 (199). Interestingly, the two visual arrestins, arrestin and cone arrestin, lack significant portions of this domain and are not very homologous with the remaining residues (Figure 2). These results correlate well with characterization of the binding domain in clathrin for nonvisual arrestins (200). In the latter study, a small region of highly conserved residues in the clathrin heavy chain N-terminal domain containing critical basic and hydrophobic residues was found to be important for arrestin binding. Thus, it is likely that ionic and hydrophobic and/or hydrogen bonding interactions contribute to high-affinity binding between nonvisual arrestins and clathrin.

Other GPR signaling systems will likely utilize a similar mechanism for regulation of signaling, whereby binding of nonvisual arrestins to the agonist-activated GRK-phosphorylated GPR results in concomitant desensitization and targeting of the GPR to clathrin-coated pits for internalization. By virtue of their ability to promote internalization of GPRs, a process now believed to be critical for recovery of responsiveness following agonist-induced desensitization,

GRKs and arrestins have now been linked to both the desensitization and resensitization of GPRs following agonist stimulation. This raises the intriguing possibility that these proteins may have other functions as well.

ACKNOWLEDGMENTS

We thank members of the Benovic laboratory for helpful discussion. JLB is an Established Investigator of the American Heart Association and is supported by NIH grants GM44944 and GM47417. JGK was supported by NIH training grant DK07705.

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Literature Cited

1. Hausdorff WP, Caron MG, Lefkowitz RJ. 1990. Turning off the signal: desensitization of β -adrenergic receptor function. *FASEB J.* 4:2881-89
2. Hargrave PA, McDowell JH. 1992. Rhodopsin and phototransduction: a model system for G protein-linked receptors. *FASEB J.* 6:2323-31
3. Schleicher A, Kuhn H, Hofmann KP. 1989. Kinetics, binding constant, and activation energy of the 48-kDa protein-rhodopsin complex by extra-metaphorhodopsin II. *Biochemistry* 28:1770-75
4. Shear M, Insel PA, Melmon KL, Coffino P. 1976. Agonist-specific refractoriness induced by isoproterenol: a study with mutant cells. *J. Biol. Chem.* 252:7572-76
5. Su Y-F, Harden TK, Perkins JP. 1979. Isoproterenol-induced desensitization of adenylate cyclase in human astrocytoma cells. *J. Biol. Chem.* 254:38-41
6. Sibley DR, Benovic JL, Caron MG, Lefkowitz RJ. 1987. Regulation of transmembrane signaling by receptor phosphorylation. *Cell* 48:913-22
7. Strulovici B, Cerione RA, Kilpatrick BF, Caron MG, Lefkowitz RJ. 1984. Direct demonstration of impaired functionality of a purified desensitized β -adrenergic receptor in a reconstitution system. *Science* 225:837-40
8. Kuhn H, Dreyer WJ. 1972. Light-dependent phosphorylation of rhodopsin by ATP. *FEBS Lett.* 20:1-6
9. Bownds D, Dawes J, Miller J, Stahlman M. 1972. Phosphorylation of frog photoreceptor membranes induced by light. *Nature* 237:125-27
10. Sitaramayya A, Liebman PA. 1983. Phosphorylation of rhodopsin and quenching of cyclic cGMP phosphodiesterase activation by ATP and weak bleaches. *J. Biol. Chem.* 258:12106-9
11. Wilden U, Kuhn H. 1982. Light-dependent phosphorylation of rhodopsin: number of phosphorylation sites. *Biochemistry* 21:3014-22
12. Kuhn H. 1978. Light-regulated binding of rhodopsin kinase and other proteins to cattle photoreceptor membranes. *Biochemistry* 17:4389-95
13. Palczewski K, McDowell JH, Hargrave PA. 1988. Purification and characterization of rhodopsin kinase. *J. Biol. Chem.* 263:14067-73
14. Fukada Y, Kokame K, Okano T, Shahida Y, Yoshizawa T, et al. 1990. Phosphorylation of iodopsin, chicken red-sensitive cone visual pigment. *Biochemistry* 29:10102-6
15. Lorenz W, Inglese J, Palczewski K, Onorato JJ, Caron MG, et al. 1991. The receptor kinase family: Primary structure of rhodopsin kinase reveals similarities to the β adrenergic receptor kinase. *Proc. Natl. Acad. Sci. USA* 88:8715-19
16. Somers RL, Klein DC. 1984. Rhodopsin kinase activity in the mammalian pineal and other tissues. *Science* 226:182-84
17. Anant JS, Fung BK. 1992. *In vivo* farnesylation of rat rhodopsin kinase. *Biochem. Biophys. Res. Commun.* 183:468-73
18. Inglese J, Glickman JF, Lorenz W, Caron MG, Lefkowitz RJ. 1992. Isoprenylation of a protein kinase: requirement of farnesylation/ α -carboxyl methylation for full enzymatic activity of rhodopsin kinase. *J. Biol. Chem.* 267:1422-25

19. Thompson P, Findlay JBC. 1984. Phosphorylation of bovine rhodopsin. *Biochem. J.* 220:773-80
20. Palczewski K, Buczylo J, Kaplan MW, Polans AS, Crabb JW. 1991. Mechanism of rhodopsin kinase activation. *J. Biol. Chem.* 266:12949-55
21. Kuhn H, Mommertz O, Hargrave PA. 1982. Light-dependent conformational change at rhodopsin's cytoplasmic surface detected by increased susceptibility to proteolysis. *Biochim. Biophys. Acta* 679:95-100
22. Papac DL, Oatis JE Jr, Crouch RK, Knapp DR. 1993. Mass spectrometric identification of phosphorylation sites in bleached bovine rhodopsin. *Biochemistry* 32:5930-34
23. Ohguro H, Palczewski K, Ericsson LH, Walsh KA, Johnson RS. 1993. Sequential phosphorylation of rhodopsin at multiple sites. *Biochemistry* 32:5718-24
24. McDowell JH, Nawrocki JP, Hargrave PA. 1993. Phosphorylation sites in bovine rhodopsin. *Biochemistry* 32:4968-74
25. Ohguro H, Van Hooser JP, Milam AH, Palczewski K. 1995. Rhodopsin phosphorylation and dephosphorylation *in vivo*. *J. Biol. Chem.* 270:14259-62
26. Ohguro H, Rudnicka-Nawrot M, Buczylo J, Zhao X, Taylor JA, et al. 1996. Structural and enzymatic aspects of rhodopsin phosphorylation. *J. Biol. Chem.* 271:5215-24
27. Fowles C, Sharma R, Akhtar M. 1988. Mechanistic studies on the phosphorylation of photoexcited rhodopsin. *FEBS Lett.* 238:56-60
28. Miller JL, Fox DA, Litman BJ. 1986. Amplification of phosphodiesterase activation is greatly reduced by rhodopsin phosphorylation. *Biochemistry* 25:4984-88
29. Chen J, Makino CL, Peachey NS, Baylor DA, Simon MI. 1995. Mechanisms of rhodopsin inactivation *in vivo* as revealed by a COOH-terminal truncation mutant. *Science* 267:374-77
30. Stadel JM, Nambi P, Lavin TN, Heald SL, Caron MG, Lefkowitz RJ. 1982. Catecholamine-induced desensitization of turkey erythrocyte adenylate cyclase. *J. Biol. Chem.* 257:9242-45
31. Stadel JM, Nambi P, Shorr RG, Sawyer DF, Caron MG, Lefkowitz RJ. 1983. Catecholamine-induced desensitization of turkey erythrocyte adenylate cyclase is associated with phosphorylation of the β -adrenergic receptor. *Proc. Natl. Acad. Sci. USA* 80:3173-77
32. Strasser RH, Sibley DR, Lefkowitz RJ. 1986. A novel catecholamine-activated adenosine 3',5'-phosphate independent pathway for β -adrenergic receptor phosphorylation in wild-type and mutant s49 lymphoma cells: mechanism of homologous desensitization of adenylate cyclase. *Biochemistry* 25:1371-77
33. Green DA, Clark RB. 1981. Adenylate cyclase-coupling proteins are not essential for agonist-specific desensitization of lymphoma cells. *J. Biol. Chem.* 256:2105-8
34. Benovic JL, Strasser RH, Caron MG, Lefkowitz RJ. 1986. β -Adrenergic receptor kinase: identification of a novel protein kinase that phosphorylates the agonist-occupied form of the receptor. *Proc. Natl. Acad. Sci. USA* 83:2797-801
35. Benovic JL, DeBlasi A, Stone WC, Caron MG, Lefkowitz RJ. 1989. β -adrenergic receptor kinase: Primary structure delineates a multigene family. *Science* 246:235-39
36. Benovic JL, Mayor F Jr, Staniszewski C, Lefkowitz RJ, Caron MG. 1987. Purification and characterization of the β -adrenergic receptor kinase. *J. Biol. Chem.* 262:9026-32
37. Benovic JL, Mayor F Jr, Somers RL, Caron MG, Lefkowitz RJ. 1986. Light-dependent phosphorylation of rhodopsin by β -adrenergic receptor kinase. *Nature* 322:869-72
38. Chen C-Y, Dion SB, Kim CM, Benovic JL. 1993. β -adrenergic receptor kinase: Agonist-dependent receptor binding promotes kinase activation. *J. Biol. Chem.* 268:7825-31
39. Benovic JL, Onorato J, Lohse MJ, Dohlman HG, Staniszewski C, et al. 1990. Synthetic peptides of the hamster β_2 -adrenoceptor as substrates and inhibitors of the β -adrenoceptor kinase. *Br. J. Pharmacol.* 30:3S-15S
40. Fredericks ZL, Pitcher JA, Lefkowitz RJ. 1996. Identification of G protein-coupled receptor kinase phosphorylation sites in the human β_2 -adrenergic receptor. *J. Biol. Chem.* 271:13795-803
41. Benovic JL, Onorato JJ, Arriza JL, Stone WC, Lohse M, et al. 1991. Cloning, expression, and chromosomal localization of β -adrenergic receptor kinase 2: a new member of the receptor kinase family. *J. Biol. Chem.* 266:14939-46
42. Ambrose C, James M, Barnes G, Lin C, Bates G, et al. 1992. A novel G protein-coupled receptor kinase gene cloned from 4p16.3. *Hum. Mol. Genet.* 1:697-703
43. Kunapuli P, Benovic JL. 1993. Cloning and expression of GRK5: a member of the G protein-coupled receptor kinase family.

- Proc. Natl. Acad. Sci. USA* 90:5588-92
44. Benovic JL, Gomez J. 1993. Molecular cloning and expression of GRK6: a new member of the G protein-coupled receptor kinase family. *J. Biol. Chem.* 268:19521-27
 45. Cassill JA, Whitney M, Joazeiro CAP, Becker A, Zuker CS. 1991. Isolation of *Drosophila* genes encoding G protein-coupled receptor kinases. *Proc. Natl. Acad. Sci. USA* 88:11067-70
 46. Kim CM, Dion SB, Onorato JJ, Benovic JL. 1993. Expression and characterization of two β -adrenergic receptor kinase isoforms using the baculovirus expression system. *Receptor* 3:39-55
 47. Haga K, Haga T. 1992. Activation by G protein $\beta\gamma$ subunits of agonist- or light-dependent phosphorylation of muscarinic acetylcholine receptors and rhodopsin. *J. Biol. Chem.* 267:2222-27
 48. Pitcher JA, Inglese J, Higgins JB, Arriza JL, Casey PJ, et al. 1992. Role of $\beta\gamma$ subunits of G proteins in targeting the β -adrenergic receptor kinase to membrane-bound receptors. *Science* 257:1264-67
 49. Kim CM, Dion SB, Benovic JL. 1993. Mechanism of β -adrenergic receptor kinase activation by G proteins. *J. Biol. Chem.* 268:15412-18
 50. DebBurman SK, Ptasienski J, Boetticher E, Lomasney JW, Benovic JL, Hosey MM. 1995. Lipid-mediated regulation of G protein-coupled receptor kinases 2 and 3. *J. Biol. Chem.* 270:5742-47
 51. Onorato JJ, Gillis ME, Liu Y, Benovic JL, Ruoho AE. 1995. The β -adrenergic receptor kinase (GRK2) is regulated by phospholipids. *J. Biol. Chem.* 270:21346-53
 52. Pitcher JA, Touhara K, Payne ES, Lefkowitz RJ. 1995. Pleckstrin homology domain-mediated membrane association and activation of the β -adrenergic receptor kinase requires coordinate interaction with $G_{\beta\gamma}$ and lipid. *J. Biol. Chem.* 270:11707-10
 53. DebBurman SK, Ptasienski J, Benovic JL, Hosey MM. 1996. G protein-coupled receptor kinase GRK2 is a phospholipid-dependent enzyme that can be conditionally activated by G protein $\beta\gamma$ subunits. *J. Biol. Chem.* 271:22552-62
 54. Koch WJ, Inglese J, Stone WC, Lefkowitz RJ. 1993. The binding site for the $\beta\gamma$ subunits of heterotrimeric G proteins on the β -adrenergic receptor kinase. *J. Biol. Chem.* 268:8256-60
 55. Premont RT, Macrae AD, Stoffel RH, Chung N, Pitcher JA, et al. 1996. Characterization of the G protein-coupled receptor kinase GRK4: identification of splice variants. *J. Biol. Chem.* 271:6403-10
 56. Stoffel RH, Randall RR, Premont RT, Lefkowitz RJ, Inglese J. 1994. Palmitoylation of G protein-coupled receptor kinase, GRK6: lipid modification diversity in the GRK family. *J. Biol. Chem.* 269:27791-94
 57. Kunapuli P, Gurevich VV, Benovic JL. 1994. Phospholipid-stimulated autophosphorylation activates the G protein-coupled receptor kinase GRK5. *J. Biol. Chem.* 269:10209-12
 58. Pitcher JA, Fredericks ZL, Stone WC, Premont RT, Stoffel RH, et al. 1996. Phosphatidylinositol 4,5-bisphosphate (PIP₂)-enhanced G protein-coupled receptor kinase (GRK) activity: Location, structure, and regulation of the PIP₂ binding site distinguishes the GRK subfamilies. *J. Biol. Chem.* 271:24907-13
 59. Loudon RP, Benovic JL. 1997. Regulation of the G protein-coupled receptor kinase GRK6: altered activity of palmitoylation-deficient and isoprenylated forms of GRK6. *J. Biol. Chem.* 272:27422-27
 60. Chen C-K, Inglese J, Lefkowitz RJ, Hurlley JB. 1995. Ca²⁺-dependent interaction of recoverin with rhodopsin kinase. *J. Biol. Chem.* 270:18060-66
 61. Yarfitz S, Hurlley JB. 1994. Transduction mechanisms of vertebrate and invertebrate photoreceptors. *J. Biol. Chem.* 269:14329-32
 62. Chuang TT, LeVine H III, DeBlasi A. 1995. Phosphorylation and activation of β -adrenergic receptor kinase by protein kinase C. *J. Biol. Chem.* 270:18660-65
 63. Winstel R, Freund S, Krasel C, Hoppe E, Lohse MJ. 1996. Protein kinase cross-talk: membrane targeting of the β -adrenergic receptor kinase by protein kinase C. *Proc. Natl. Acad. Sci. USA* 93:2105-9
 64. Pronin AN, Benovic JL. 1997. Regulation of the G protein-coupled receptor kinase GRK5 by protein kinase C. *J. Biol. Chem.* 272:3806-12
 65. Chuang TT, Paolucci L, DeBlasi A. 1996. Inhibition of G protein-coupled receptor kinase subtypes by Ca²⁺/calmodulin. *J. Biol. Chem.* 271:28691-96
 66. Haga K, Tsuga H, Haga T. 1997. Ca²⁺-dependent inhibition of G protein-coupled receptor kinase 2 by calmodulin. *Biochemistry* 36:1315-21
 67. Pronin AN, Satpaev DK, Slepak VZ, Benovic JL. 1997. Regulation of G protein-coupled receptor kinases by calmodulin

- and localization of the calmodulin binding domain. *J. Biol. Chem.* 272:18273-80
68. Haga K, Kameyama K, Haga T, Kikkawa U, Shiozaki K, Uchiyama H. 1996. Phosphorylation of human m1 muscarinic acetylcholine receptors by G protein-coupled receptor kinase 2 and protein kinase C. *J. Biol. Chem.* 271:2776-82
 69. Richardson RM, Kim C, Benovic JL, Hosey MM. 1993. Phosphorylation and desensitization of human m2 muscarinic acetylcholine receptors by two isoforms of the β -adrenergic receptor kinase. *J. Biol. Chem.* 268:13650-56
 70. DebBurman SK, Kunapuli P, Benovic JL, Hosey MM. 1995. Agonist-dependent phosphorylation of human muscarinic receptors in *Spodoptera frugiperda* insect cell membranes by G protein-coupled receptor kinases. *Mol. Pharmacol.* 47:224-33
 71. Benovic JL, Regan JW, Matsui H, Mayor F Jr, Cottechia S, et al. 1987. Agonist-dependent phosphorylation of the α_2 -adrenergic receptor by the β -adrenergic receptor kinase. *J. Biol. Chem.* 262:17251-53
 72. Opperman M, Freedman NJ, Alexander RW, Lefkowitz RJ. 1996. Phosphorylation of the type 1A angiotensin II receptor by G protein-coupled receptor kinases and protein kinase C. *J. Biol. Chem.* 271:13266-72
 73. Kwatra MM, Schwinn DA, Schreurs J, Blank JL, Krause J, et al. 1993. The substance P receptor, which couples to Gq/11, is a substrate of β -adrenergic receptor kinase 1 and 2. *J. Biol. Chem.* 268:9161-64
 74. Strasser RH, Benovic JL, Caron MG, Lefkowitz RJ. 1986. Agonist- and prostaglandin E_1 -induced translocation of the β -adrenergic receptor kinase: Evidence that the kinase may act on multiple adenylate cyclase-coupled receptors. *Proc. Natl. Acad. Sci. USA* 83:6362-66
 75. Mayor F Jr, Benovic JL, Caron MG, Lefkowitz RJ. 1987. Somatostatin induces translocation of the β -adrenergic receptor kinase and desensitizes somatostatin receptors in s49 lymphoma cells. *J. Biol. Chem.* 262:6468-71
 76. Boekhoff I, Inglese J, Schleicher S, Koch WJ, Lefkowitz RJ, Breer H. 1994. Olfactory desensitization requires membrane targeting of receptor kinase mediated by $\beta\gamma$ -subunits of heterotrimeric G proteins. *J. Biol. Chem.* 269:37-40
 77. Dawson TM, Arriza JL, Jaworsky DE, Borisy FF, Attramadal H, et al. 1993. β -adrenergic receptor kinase-2 and β -arrestin2 as mediators of odorant-induced desensitization. *Science* 259:825-29
 78. Schleicher S, Boekhoff I, Arriza J, Lefkowitz RJ, Breer H. 1993. A β -adrenergic kinase-like enzyme is involved in olfactory signal termination. *Proc. Natl. Acad. Sci. USA* 90:1420-24
 79. Jewell-Motz E, Liggett SB. 1996. G protein-coupled receptor kinase specificity for phosphorylation and desensitization of α_2 -adrenergic receptor subtypes. *J. Biol. Chem.* 271:18082-87
 80. Opperman M, Diverse-Pierluissi M, Drazner MH, Dyer SL, Freedman NJ, et al. 1996. Monoclonal antibodies reveal receptor specificity among G-protein-coupled receptor kinases. *Proc. Natl. Acad. Sci. USA* 93:7649-54
 81. Pippig S, Andexinger S, Daniel K, Puzicha M, Caron MG, et al. 1993. Overexpression of β -arrestin and β -adrenergic receptor kinase augment desensitization of β_2 -adrenergic receptor. *J. Biol. Chem.* 268:3201-8
 82. Freedman NJ, Liggett SB, Drachman DE, Pei G, Caron MG, Lefkowitz RJ. 1995. Phosphorylation and desensitization of the human β_1 -adrenergic receptor: involvement of G protein-coupled receptor kinases and cAMP-dependent protein kinase. *J. Biol. Chem.* 270:17953-61
 83. Diviani D, Lattion A-L, Larbi N, Kunapuli P, Pronin A, et al. 1996. Effect of different G protein-coupled receptor kinases on phosphorylation and desensitization of the α_{1B} -adrenergic receptor. *J. Biol. Chem.* 271:5049-58
 84. Ishii K, Chen J, Ishii M, Koch WJ, Freedman NJ, et al. 1994. Inhibition of thrombin receptor signaling by a G protein-coupled receptor kinase: functional specificity among G protein-coupled receptor kinases. *J. Biol. Chem.* 269:1125-30
 85. Palmer TM, Benovic JL, Stiles GL. 1995. Agonist-dependent phosphorylation and desensitization of the rat A3 adenosine receptor: evidence for a G protein-coupled receptor kinase-mediated mechanism. *J. Biol. Chem.* 270:29607-13
 86. Schlador ML, Nathanson NM. 1997. Synergistic regulation of m2 muscarinic acetylcholine receptor desensitization and sequestration by G protein-coupled receptor kinase-2 and β -arrestin-1. *J. Biol. Chem.* 272:18882-90
 87. Liggett SB, Ostrowski J, Chesnut LC, Kurose H, Raymond JR, et al. 1992. Sites in the third intracellular loop of the α_{2A} -adrenergic receptor confer short term agonist-promoted desensitization:

- evidence for a receptor kinase-mediated mechanism. *J. Biol. Chem.* 267:4740-46
88. Pals-Rylaarsdam R, Xu Y, Witt-Enderby P, Benovic JL, Hosey MM. 1995. Desensitization and internalization of the m2 muscarinic acetylcholine receptor are directed by independent mechanisms. *J. Biol. Chem.* 270:29004-11
 89. Bouvier M, Hausdorff WP, DeBlasi A, O'Dowd BF, Kobilka BK, et al. 1988. Removal of phosphorylation sites from the β_2 -adrenergic receptor delays onset of agonist-promoted desensitization. *Nature* 333:370-73
 90. Sanchez-Yague J, Rodriguez MC, Segaloff IM, Ascoli M. 1992. Truncation of the cytoplasmic tail of the lutropin/choriogonadotropin receptor prevents agonist-induced coupling. *J. Biol. Chem.* 267:7217-20
 91. Alblas J, van Etten I, Khanum A, Mooleenaar WH. 1995. C-terminal truncation of the neurokinin-2 receptor causes enhanced and sustained agonist-induced signaling: role of receptor phosphorylation in signal attenuation. *J. Biol. Chem.* 270:8944-51
 92. Lattion A-L, Diviani D, Cotecchia S. 1994. Truncation of the receptor carboxyl terminus impairs agonist-dependent phosphorylation and desensitization of the α_{1B} -adrenergic receptor. *J. Biol. Chem.* 269:22887-93
 93. Takano T, Honda Z, Sakanaka C, Izumi T, Kameyama K, et al. 1994. Role of cytoplasmic tail phosphorylation sites of platelet-activating factor receptor in agonist-induced desensitization. *J. Biol. Chem.* 269:22453-58
 94. Lohse MJ, Benovic JL, Caron MG, Lefkowitz RJ. 1990. Multiple pathways of rapid β_2 -adrenergic receptor desensitization: delineation with specific inhibitors. *J. Biol. Chem.* 265:3202-9
 95. Kong G, Penn R, Benovic JL. 1994. A β -adrenergic receptor kinase dominant-negative mutant attenuates desensitization of the β_2 -adrenergic receptor. *J. Biol. Chem.* 269:13084-87
 96. Pei G, Kieffer BL, Lefkowitz RJ, Freedman NJ. 1995. Agonist-dependent phosphorylation of the mouse δ -opioid receptor: involvement of G protein-coupled receptor kinases but not protein kinase C. *Mol. Pharmacol.* 48:173-77
 97. Mundell SJ, Benovic JL, Kelly E. 1997. A dominant-negative mutant of the G protein-coupled receptor kinase 2 selectively attenuates adenosine A_2 receptor desensitization. *Mol. Pharmacol.* 51:991-98
 98. Vaughan RA, Devreotes PN. 1988. Ligand-induced phosphorylation of the cAMP receptor from *Dictyostelium discoideum*. *J. Biol. Chem.* 263:14538-43
 99. Reneke JE, Blumer KJ, Courchesne WE, Thorner J. 1988. The carboxyl-terminal segment of the yeast a-factor receptor is a regulatory domain. *Cell* 55:221-28
 100. Koch WJ, Rockman HA, Samama P, Hamilton R, Bond RA, et al. 1995. Cardiac function in mice overexpressing the β -adrenergic receptor kinase or a β ARK inhibitor. *Science* 268:1350-53
 101. Rockman HA, Choi D-J, Rahman NU, Akhter SA, Lefkowitz RJ, Koch WJ. 1996. Receptor-specific *in vivo* desensitization by the G protein-coupled receptor kinase-5 in transgenic mice. *Proc. Natl. Acad. Sci. USA* 93:9954-59
 102. Shih M, Malbon CC. 1994. Oligodeoxynucleotides antisense to mRNA encoding protein kinase A, protein kinase C, and β -adrenergic receptor kinase reveal distinctive cell-type-specific roles in agonist-induced desensitization. *Proc. Natl. Acad. Sci. USA* 91:12193-97
 103. Nakata H, Kinoshita Y, Kishi K, Fukada H, Kawanami C, et al. 1996. Involvement of β -adrenergic receptor kinase-1 in homologous desensitization of histamine H_2 receptors in human gastric carcinoma cell line MKN-45. *Digestion* 57:406-10
 104. Nagayama Y, Takeshi K, Hara T, Namba H, Yamashita S, et al. 1996. Involvement of G protein-coupled receptor kinase 5 in homologous desensitization of the thyrotropin receptor. *J. Biol. Chem.* 271:10143-48
 105. Pfister C, Chabre M, Plouet J, Tuyen VV, DeKozak Y, et al. 1985. Retinal S antigen identified as the 48K protein regulating light-dependent phosphodiesterase in rods. *Science* 228:891-93
 106. Kuhn H, Hall SW, Wilden U. 1984. Light-induced binding of 48-kDa protein to photoreceptor membranes is highly enhanced by phosphorylation of rhodopsin. *FEBS Lett.* 176:473-78
 107. Wilden U, Wust E, Weyand I, Kuhn H. 1986. Rapid affinity purification of retinal arrestin (48-kDa protein) via its light-dependent binding to phosphorylated rhodopsin. *FEBS Lett.* 207:292-95
 108. Yamaki K, Takahashi Y, Sakuragi S, Matsumura K. 1987. Molecular cloning of the S-antigen cDNA from bovine retina. *Biochem. Biophys. Res. Commun.* 142:904-10
 109. Shinohara T, Dietzschold B, Craft CM, Wistow G, Early JJ, et al. 1987. Primary and secondary structure of bovine retinal

- S antigen (48-kDa protein). *Proc. Natl. Acad. Sci. USA* 84:6975-79
110. Lohse MJ, Benovic JL, Codina J, Caron MG, Lefkowitz RJ. 1990. β -arrestin: a protein that regulates β -adrenergic receptor function. *Science* 248:1547-50
 111. Murakami A, Yajima T, Sakuma H, McLaren MJ, Inani G. 1993. X-Arrestin: a new retinal arrestin mapping to the X chromosome. *FEBS Lett.* 334:203-9
 112. Craft CM, Whitmore DH, Wiechmann AF. 1994. Cone arrestin identified by targeting expression of a functional family. *J. Biol. Chem.* 269:4613-19
 113. Hyde DR, Mecklenberg KL, Pollack JA, Vihtelic TS, Benzer S. 1990. Twenty *Drosophila* visual system cDNA clones: One is a homolog of human arrestin. *Proc. Natl. Acad. Sci. USA* 87:1008-12
 114. Smith DP, Sheih BH, Zuker CS. 1990. Isolation and structure of an arrestin gene from *Drosophila*. *Proc. Natl. Acad. Sci. USA* 87:1003-7
 115. Gurevich VV, Benovic JL. 1992. Cell-free expression of visual arrestin: Truncation mutagenesis identifies multiple domains involved in rhodopsin interaction. *J. Biol. Chem.* 267:21919-23
 116. Gurevich VV, Richardson RM, Kim CM, Hosey MM, Benovic JL. 1993. Binding of wild type and chimeric arrestins to the m2 muscarinic acetylcholine receptor. *J. Biol. Chem.* 268:16879-82
 117. Gurevich VV, Dion SB, Onorato JJ, Ptasinski J, Kim CM, et al. 1995. Arrestin interactions with G protein-coupled receptors: direct binding studies of wild type and mutant arrestins with rhodopsin, β_2 -adrenergic, and m2 muscarinic cholinergic receptors. *J. Biol. Chem.* 270:720-31
 118. Wilden U, Hall SW, Kuhn H. 1986. Phosphodiesterase activation by photoexcited rhodopsin is quenched when rhodopsin is phosphorylated and binds the intrinsic 48-kDa protein of rod outer segments. *Proc. Natl. Acad. Sci. USA* 83:1174-78
 119. Bennett N, Sitarayya A. 1988. Inactivation of photoexcited rhodopsin in retinal rods: the roles of rhodopsin kinase and 48-kDa protein (arrestin). *Biochemistry* 27:1710-15
 120. Krupnick JG, Gurevich VV, Benovic JL. 1997. Mechanism of quenching of phototransduction: binding competition between arrestin and transducin for phosphorhodopsin. *J. Biol. Chem.* 272:18125-31
 121. Lohse MJ, Andexinger S, Pitcher J, Trukawinski S, Codina J, et al. 1992. Receptor-specific desensitization with purified proteins: kinase dependence and receptor specificity of β -arrestin and arrestin in the β_2 -adrenergic and rhodopsin systems. *J. Biol. Chem.* 267:8558-64
 122. Palczewski K, Buczylo J, Ohguro H, Annan RS, Carr SA, et al. 1994. Characterization of a truncated form of arrestin isolated from bovine rod outer segments. *Protein Sci.* 3:319-29
 123. Smith WC, Milam AH, Dugger D, Arendt A, Hargrave PA, Palczewski K. 1994. A splice variant of arrestin: molecular cloning and localization in bovine retina. *J. Biol. Chem.* 269:15407-10
 124. Dolph PJ, Ranganathan R, Colley NJ, Hardy RW, Socolich M, Zuker CS. 1993. Arrestin function in inactivation of G protein-coupled receptor rhodopsin in vivo. *Science* 260:1910-16
 125. Xu J, Dodd RL, Makino CL, Simon MI, Baylor DA, Chen J. 1996. Prolonged photoresponses in transgenic mouse rods lacking arrestin. *Nature* 389:505-9
 126. Rim J, Oprian DD. 1995. Constitutive activation of opsin: interaction of mutants with rhodopsin kinase and arrestin. *Biochemistry* 34:11938-45
 127. Benovic JL, Kuhn H, Weyand I, Codina J, Caron MG, Lefkowitz RJ. 1987. Functional desensitization of the isolated β -adrenergic receptor by the β -adrenergic receptor kinase: potential role of an analog of the retinal protein arrestin (48-kDa protein). *Proc. Natl. Acad. Sci. USA* 84:8879-82
 128. Sterne-Marr R, Gurevich VV, Goldsmith P, Bodine RC, Sanders C, et al. 1993. Polypeptide variants of β -arrestin and arrestin3. *J. Biol. Chem.* 268:15640-48
 129. Rapoport B, Kaufman KD, Chazenbalk GD. 1992. Cloning of a member of the arrestin family from a human thyroid cDNA library. *Mol. Cell. Endocrin.* 84:R39-R43
 130. Attramadal H, Arriza JL, Aoki C, Dawson TM, Codina J, et al. 1992. β -arrestin2, a novel member of the arrestin/ β -arrestin gene family. *J. Biol. Chem.* 267:17882-90
 131. Sohlmann P, Hekman M, Puzicha M, Buchen C, Lohse MJ. 1995. Binding of purified recombinant β -arrestin to guanine nucleotide binding protein-coupled receptors. *Eur. J. Biochem.* 232:464-72
 132. Pitcher JA, Lohse MJ, Codina J, Caron MG, Lefkowitz RJ. 1992. Desensitization of the isolated β_2 -adrenergic receptor by β -adrenergic receptor kinase, cAMP-dependent protein kinase, and protein kinase C occurs via distinct molecular mechanisms. *Biochemistry* 31:3193-97

133. Benovic JL, Pike LJ, Cerione RA, Staniszewski C, Yoshimasa T, et al. 1985. Phosphorylation of the mammalian β -adrenergic receptor by cAMP-dependent protein kinase: regulation of the rate of receptor phosphorylation and dephosphorylation by agonist occupancy and effects on coupling of receptor to stimulatory guanine nucleotide regulatory protein. *J. Biol. Chem.* 260:7094-101
134. Gurevich VV, Benovic JL. 1993. Visual arrestin interaction with rhodopsin: Sequential multisite binding ensures strict selectivity toward light-activated phosphorylated rhodopsin. *J. Biol. Chem.* 268:11628-38
135. Krupnick JG, Gurevich VV, Schepers T, Hamm HE, Benovic JL. 1994. Arrestin-rhodopsin interaction: multi-site binding delineated by peptide inhibition. *J. Biol. Chem.* 269:3226-32
136. Wu G, Krupnick JG, Benovic JL, Lanier SM. 1997. Interaction of arrestins with intracellular domains of muscarinic and α_2 -adrenergic receptors. *J. Biol. Chem.* 272:17836-42
137. Palczewski K, Pulvermiller A, Buczylo J, Hofmann KP. 1991. Phosphorylated rhodopsin and heparin induce similar conformational changes in arrestin. *J. Biol. Chem.* 266:18649-54
138. Kieselbach T, Irrgang K-D, Ruppel H. 1994. A segment corresponding to amino acids Val170-Arg182 of bovine arrestin is capable of binding to phosphorylated rhodopsin. *Eur. J. Biochem.* 226:87-97
139. Gurevich VV, Benovic JL. 1995. Visual arrestin binding to rhodopsin: diverse functional roles of positively charged residues within the phosphorylation-recognition region of arrestin. *J. Biol. Chem.* 270:6010-16
140. Puig J, Arendt A, Tomson FL, Abdulaeva G, Miller R, et al. 1995. Synthetic phosphopeptide from rhodopsin sequence induces retinal arrestin binding to photoactivated unphosphorylated rhodopsin. *FEBS Lett.* 362:185-88
141. Palczewski K, Buczylo J, Imami NR, McDowell JH, Hargrave PA. 1991. Role of the carboxyl-terminal region of arrestin in binding to phosphorylated rhodopsin. *J. Biol. Chem.* 266:15334-39
142. Gurevich VV, Chen C-Y, Kim CM, Benovic JL. 1994. Visual arrestin binding to rhodopsin: Intramolecular interaction between the basic N terminus and acidic C terminus of arrestin may regulate binding selectivity. *J. Biol. Chem.* 269:8721-27
143. Chuang D-M, Costa E. 1979. Evidence for internalization of the recognition site of β -adrenergic receptors during receptor subsensitivity induced by (-)-isoproterenol. *Proc. Natl. Acad. Sci. USA* 76:3024-28
144. Pittman RN, Molinoff PB. 1980. Interactions of agonists and antagonists with β -adrenergic receptors in intact L6 muscle cells. *J. Cyclic Nucleotide Protein Phosphor. Res.* 6:421-35
145. Toews ML, Harden TK, Perkins JP. 1983. High-affinity binding of agonists to β -adrenergic receptors on intact cells. *Proc. Natl. Acad. Sci. USA* 80:3553-57
146. Staehelin M, Simons P. 1982. Rapid and reversible disappearance of β -adrenergic cell surface receptors. *EMBO J.* 1:187-90
147. Harden TK, Cotton CU, Waldo GL, Lutton JK, Perkins JP. 1980. Catecholamine-induced alteration in sedimentation behavior of membrane bound β -adrenergic receptors. *Science* 210:441-43
148. Toews ML, Waldo GL, Harden TK, Perkins JP. 1984. Relationship between an altered membrane form and a low-affinity form of the β -adrenergic receptor occurring during catecholamine-induced desensitization: evidence for receptor internalization. *J. Biol. Chem.* 259:11844-50
149. Waldo GL, Northup JK, Perkins JP, Harden TK. 1983. Characterization of an altered membrane form of the β -adrenergic receptor produced during agonist-induced desensitization. *J. Biol. Chem.* 258:13900-8
150. Clark RB, Friedman J, Prashad N, Ruoho AE. 1985. Epinephrine-induced sequestration of the β -adrenergic receptor in cultured s49 wt and cyc-lymphoma cells. *J. Cyclic Nucleotide Protein Phosphor. Res.* 10:97-119
151. von Zastrow M, Kobilka BK. 1992. Ligand-regulated internalization and recycling of human β_2 -adrenergic receptors between the plasma membrane and endosomes containing transferrin receptors. *J. Biol. Chem.* 267:3530-38
152. Wakshull E, Hertel C, O'Keefe EJ, Perkins JP. 1985. Cellular redistribution of β -adrenergic receptors in a human astrocytoma cell line: a comparison with the epidermal growth factor receptor in murine fibroblasts. *J. Cell Biol.* 29:127-41
153. Homburger V, Lucas M, Cantau R, Barabe J, Penit J, Bockaert J. 1980. Further evidence that desensitization of β -adrenergic-sensitive adenylate cyclase proceeds in two steps: modification of the coupling and loss of β -adrenergic receptors. *J. Biol. Chem.* 255:10436-44

154. Chuang D-M, Kinnier WJ, Farber L, Costa E. 1980. A biochemical study of receptor internalization during β -adrenergic receptor desensitization in frog erythrocytes. *Mol. Pharmacol.* 18:348-55.
155. Hertel C, Coulter SJ, Perkins JP. 1985. A comparison of catecholamine-induced internalization of β -adrenergic receptors and receptor-mediated endocytosis of epidermal growth factor in human astrocytoma cells. *J. Biol. Chem.* 260:12547-53.
156. Kurz JB, Perkins JP. 1992. Isoproterenol-initiated β -adrenergic receptor diacytosis in cultured cells. *Mol. Pharmacol.* 41:375-81.
157. Koenig JA, Edwardson JM. 1996. Intracellular trafficking of the muscarinic acetylcholine receptor: importance of subtype and cell type. *Mol. Pharmacol.* 49:351-59.
158. Keith DE, Murray SR, Zaki PA, Chu PC, Lissin DV, et al. 1996. Morphine activates opioid receptors without causing their rapid internalization. *J. Biol. Chem.* 271:19021-24.
159. Berry SA, Shah MC, Khan N, Roth BL. 1996. Rapid agonist-induced internalization of the 5-hydroxytryptamine_{2A} receptor occurs via the endosome pathway in vitro. *Mol. Pharmacol.* 50:306-13.
160. Yang J, Williams JA, Yule DI, Logsdon CD. 1995. Mutation of carboxyl-terminal threonine residues in human m3 muscarinic acetylcholine receptor modulates the extent of sequestration and desensitization. *Mol. Pharmacol.* 48:477-85.
161. Slowiejko DM, McEwen EL, Ernst SA, Fisher SK. 1996. Muscarinic receptor sequestration in SH-SY5Y neuroblastoma cells is inhibited when clathrin distribution is perturbed. *J. Neurochem.* 66:186-96.
162. Trapaidze N, Keith DE, Cvejic S, Evans CJ, Devi LA. 1996. Sequestration of the δ -opioid receptor: role of the C-terminus in agonist-mediated internalization. *J. Biol. Chem.* 271:29279-85.
163. Fonseca MI, Button DC, Brown RD. 1995. Agonist regulation of α_{1B} -adrenergic receptor subcellular distribution and function. *J. Biol. Chem.* 270:8902-9.
164. Tan PK, Davis NG, Sprague GF, Payne GS. 1993. Clathrin facilitates the internalization of seven transmembrane segment receptors forming pheromones in yeast. *J. Cell Biol.* 123:1707-16.
165. Strasser RH, Stiles GL, Lefkowitz RJ. 1984. Translocation and uncoupling of the β -adrenergic receptor in rat lung after catecholamine-promoted desensitization in vivo. *Endocrinology* 115:1392-400.
166. Mickey JV, Tate R, Mullikan D, Lefkowitz RJ. 1975. Regulation of adenylate cyclase-coupled β -adrenergic receptor binding sites by β -adrenergic catecholamines in vitro. *Mol. Pharmacol.* 12:409-19.
167. Pippig S, Andexinger S, Lohse MJ. 1995. Sequestration and recycling of β_2 -adrenergic receptors permit receptor resensitization. *Mol. Pharmacol.* 47:666-76.
168. Roth N, Campbell PT, Caron MG, Lefkowitz RJ, Lohse MJ. 1991. Comparative rates of desensitization of β -adrenergic receptors by the β -adrenergic receptor kinase and the cyclic AMP-dependent protein kinase. *Proc. Natl. Acad. Sci. USA* 88:6201-4.
169. Pak Y, Kouvelas A, Scheideler MA, Rasmussen J, O'Dowd BF, George SR. 1996. Agonist-induced functional desensitization of the μ -opioid receptor is mediated by loss of membrane receptors rather than uncoupling from G protein. *Mol. Pharmacol.* 50:1214-22.
170. Thomas WG, Thekkumkara TJ, Motel TJ, Baker KM. 1995. Stable expression of a truncated AT_{1A} receptor in CHO-K1 cells: The carboxyl-terminal region directs agonist-induced internalization but not receptor signaling or desensitization. *J. Biol. Chem.* 270:207-13.
171. Ng GYK, Trogadis J, Stevens J, Bouverier M, O'Dowd BF, George SR. 1995. Agonist-induced desensitization of dopamine D1 receptor-stimulated adenylyl cyclase activity is temporally and biochemically distinct from D1 receptor internalization. *Proc. Natl. Acad. Sci. USA* 92:10157-61.
172. Holtmann MH, Roettger BF, Pinon DI, Miller LJ. 1996. Role of receptor phosphorylation in desensitization and internalization of the secretin receptor. *J. Biol. Chem.* 271:23566-71.
173. Garland AM, Grady EF, Lovett M, Vigna SR, Frucht MM, et al. 1996. Mechanisms of desensitization and resensitization of G protein-coupled neurokinin₁ and neurokinin₂ receptors. *Mol. Pharmacol.* 49:438-46.
174. Fukushima Y, Asano T, Takata K, Kunaki M, Ogihara T, et al. 1997. Role of the C-terminus in histamine H2 receptor signaling, desensitization, and agonist-induced internalization. *J. Biol. Chem.* 272:19464-70.
175. Su Y-F, Harden TK, Perkins JP. 1980.

- Catecholamine-specific desensitization of adenylate cyclase: evidence for a multistep process. *J. Biol. Chem.* 255: 7410-19
176. Morrison KJ, Moore RH, Carsrud NDY, Trial J, Millman EE, et al. 1996. Repetitive endocytosis and recycling of the β_2 -adrenergic receptor during agonist-induced steady state redistribution. *Mol. Pharmacol.* 50:692-99
 177. Stadel JM, Strulovici B, Nambi P, Lavin TN, Briggs MM, et al. 1983. Desensitization of the β -adrenergic receptor of frog erythrocytes: recovery and characterization of the down-regulated receptors in sequestered vesicles. *J. Biol. Chem.* 258:3032-38
 178. Strulovici B, Stadel JM, Lefkowitz RJ. 1983. Functional integrity of desensitized β -adrenergic receptors: Internalized receptors reconstitute catecholamine-stimulated adenylate cyclase activity. *J. Biol. Chem.* 258:6410-14
 179. Yu SS, Lefkowitz RJ. 1993. β -adrenergic receptor sequestration: a potential mechanism of receptor resensitization. *J. Biol. Chem.* 268:337-41
 180. Sibley DR, Strasser RH, Benovic JL, Daniel K, Lefkowitz RJ. 1986. Phosphorylation/dephosphorylation of the β -adrenergic receptor regulates its functional coupling to adenylate cyclase and subcellular distribution. *Proc. Natl. Acad. Sci. USA* 83:9408-12
 181. Pitcher JA, Payne ES, Csontos C, DePaoli-Roach AA, Lefkowitz RJ. 1995. The G-protein-coupled receptor phosphatase: a protein phosphatase type 2A with a distinct subcellular distribution and substrate specificity. *Proc. Natl. Acad. Sci. USA* 92:8343-47
 182. Krueger KM, Daaka Y, Pitcher JA, Lefkowitz RJ. 1997. The role of sequestration in G protein-coupled receptor resensitization: regulation of β_2 -adrenergic receptor dephosphorylation by vesicular acidification. *J. Biol. Chem.* 272: 5-8
 183. Hein L, Ishii K, Coughlin SR, Kobilka BK. 1994. Intracellular targeting and trafficking of thrombin receptors: a novel mechanism for resensitization of a G protein-coupled receptor. *J. Biol. Chem.* 269:27719-26
 184. Palczewski K, McDowell JH, Jakes S, Ingebritsen TS, Hargrave PA. 1989. Regulation of rhodopsin dephosphorylation by arrestin. *J. Biol. Chem.* 264:15770-73
 185. Hofmann KP, Pulvermüller A, Buczylo J, VanHooser P, Palczewski K. 1992. The role of arrestins and retinoids in the regeneration pathway of rhodopsin. *J. Biol. Chem.* 267:15701-6
 186. Strader CD, Sigal IS, Blake AD, Cheung AH, Register RB, et al. 1987. The carboxyl terminus of the hamster β -adrenergic receptor expressed in mouse L cells is not required for receptor sequestration. *Cell* 49:855-63
 187. Cheung AH, Sigal IS, Dixon RAF, Strader CD. 1989. Agonist-promoted sequestration of the β_2 -adrenergic receptor requires regions involved in functional coupling with G_s . *Mol. Pharmacol.* 34:132-38
 188. Hausdorff WP, Campbell PT, Ostrowski J, Yu SS, Caron MG, Lefkowitz RJ. 1991. A small region of the β -adrenergic receptor is selectively involved in its rapid regulation. *Proc. Natl. Acad. Sci. USA* 88:2979-83
 189. Moro O, Lameh J, Sadee W. 1993. Serine- and threonine-rich domain regulates internalization of muscarinic cholinergic receptors. *J. Biol. Chem.* 268:6862-65
 190. Shapiro MJ, Trejo J, Zeng D, Coughlin SR. 1996. Role of the thrombin receptor's cytoplasmic tail in intracellular trafficking: distinct determinants for agonist-triggered versus tonic internalization and intracellular localization. *J. Biol. Chem.* 271:32874-80
 191. Tsuga H, Kameyama K, Haga T, Kurose H, Nagao T. 1994. Sequestration of muscarinic acetylcholine receptor m2 subtypes: facilitation by G protein-coupled receptor kinase (GRK2) and attenuation by a dominant-negative mutant of GRK2. *J. Biol. Chem.* 269:32522-27
 192. Barak LS, Tiberi M, Freedman NJ, Kwatra MM, Lefkowitz RJ, Caron MG. 1994. A highly conserved tyrosine residue in G protein-coupled receptors is required for agonist-mediated β_2 -adrenergic receptor sequestration. *J. Biol. Chem.* 269:2790-95
 193. Ferguson SSG, Menard L, Barak LS, Koch WJ, Colapietro A-M, Caron MG. 1995. Role of phosphorylation in agonist-promoted β_2 -adrenergic receptor sequestration: rescue of a sequestration-defective mutant receptor by β ARK1. *J. Biol. Chem.* 270:24782-89
 194. Menard L, Ferguson SSG, Barak LS, Bertrand L, Premont RT, et al. 1996. Members of the G protein-coupled receptor kinase family that phosphorylate the β_2 -adrenergic receptor facilitate sequestration. *Biochemistry* 35:4155-60
 195. Ferguson SSG, Downey WE III, Colapietro A-M, Barak LS, Menard L, Caron MG. 1996. Role of β -arrestin in

- mediating agonist-promoted G protein-coupled receptor internalization. *Science* 271:363-65
196. Menard L, Ferguson SSG, Zhang J, Lin F-T, Lefkowitz RJ, et al. 1997. Synergistic regulation of β_2 -adrenergic receptor sequestration: Intracellular complement of β -adrenergic receptor kinase and β -arrestin determine kinetics of internalization. *Mol. Pharmacol.* 51:800-8
197. Zhang J, Ferguson SSG, Barak LS, Menard L, Caron MG. 1996. Dynamin and β -arrestin reveal distinct mechanisms for G protein-coupled receptor internalization. *J. Biol. Chem.* 271:18302-5
198. Goodman OB Jr, Krupnick JG, Santini F, Gurevich VV, Penn RB, et al. 1996. β -arrestin acts as a clathrin adaptor in endocytosis of the β_2 -adrenergic receptor. *Nature* 383:447-50
199. Krupnick JG, Goodman OB Jr, Keen JH, Benovic JL. 1997. Arrestin/clathrin interaction: localization of the clathrin binding domain of nonvisual arrestins to the carboxyl terminus. *J. Biol. Chem.* 272:15011-16
200. Goodman OB Jr, Krupnick JG, Gurevich VV, Benovic JL, Keen JH. 1997. Arrestin/clathrin interaction: localization of the arrestin binding locus to the clathrin terminal domain. *J. Biol. Chem.* 272:15017-22